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<p>(54) Title: <b>A NOVEL RET-INDEPENDENT SIGNALING PATHWAY FOR GDNF</b></p> <p>(57) Abstract</p> <p>Methods for screening for agonists and antagonists of GPI-anchored independent intracellular signaling resulting in <math>[Ca^{2+}]</math> elevation, ERK1, ERK2 and CREB phosphorylation, and Src family kinase activation are described, as well as methods for treatment involving administration of agonists/antagonists of such signaling.</p> <div data-bbox="933 1144 1372 1921"> <p>The diagram illustrates a signaling pathway initiated by GDNF. Panel A shows the cell membrane with Src type kinases and GPI-anchored receptors (GFRα2, GPI link, Rafts). Panel B shows the addition of GDNF. Panel C shows the activation of Src type kinases, leading to MAPK phosphorylation and nuclear translocation (CR, EB) and PLCγ phosphorylation, resulting in IP3 production and Ca2+ release.</p> </div>		

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**A NOVEL RET-INDEPENDENT SIGNALING PATHWAY FOR GDNF****5 REFERENCE TO RELATED APPLICATIONS**

This application claims priority under 35 U.S.C. § 119(e) to U.S. provisional application 60/102,647 filed October 1, 1998, incorporated herein by reference in its entirety.

**10 FIELD OF THE INVENTION**

The present invention relates to methods for screening for agonists and antagonists of Ret-independent intracellular signaling.

**BACKGROUND OF THE INVENTION**

15 Glial cell line - derived neurotrophic factor (GDNF) (Lin et al., 1993), neurturin (NTN) (Kotzbauer et al., 1996), persephin (PSP) (Milbrandt et al., 1998) and a recently discovered artemin (ART) (Baloh et al., 1998) form a group of TGF- $\beta$  family-related neurotrophic proteins. Studies in primary neuronal cultures, as well as in lesioned animal models, have provided evidence that GDNF is a survival factor for embryonic midbrain  
20 dopaminergic neurons (Beck et al., 1995; Lin et al., 1993; Tomac et al., 1995), spinal motor neurons (Henderson et al., 1994; Oppenheim et al., 1995; Yan et al., 1995), locus coeruleus noradrenergic neurons (Arenas et al., 1995), and subpopulations of peripheral sensory, sympathetic, and parasympathetic neurons (Buj-Bello et al., 1995; Trupp et al., 1995; reviewed by Airaksinen et al., 1999 and Saarma & Sariola, 1999). The pattern of  
25 neurotrophic activity of GDNF is therefore promising for its potential use in the treatment of Parkinson disease, Alzheimer disease, motoneuron diseases and several other neurodegenerative diseases. The biological importance of the GDNF family is illustrated by the phenotype of GDNF null mice which display deficits in primary sensory, sympathetic and motor neurons. These mice also fail to develop kidneys and most of the enteric nervous  
30 system and they die at birth (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996).

Despite the potential clinical importance of the GDNF receptor family, the intracellular mechanism of GDNF's action is far from understood. Generally GDNF has been thought to act through a multi-component receptor system including a glycosyl-phosphatidyl-inositol (GPI)-anchored GDNF family receptor  $\alpha 1$  (GFR $\alpha 1$ ) (Jing et al., 1996; Treanor et al., 1996) and a transmembrane receptor tyrosine kinase, Ret (Durbec et al., 1996; Trupp et al., 1996). GFR $\alpha 1$ , lacking an intracellular domain, has originally been assessed as a binding site for GDNF, serving only in the presentation of the GFR $\alpha 1$ /GDNF complex to Ret (Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1997). There is no doubt that the Ret and GPI-anchored GFR $\alpha 1$  are necessary receptors for GDNF (Cacalano et al., 1998; Enomoto et al., 1998) since mice lacking Ret, GDNF or GFR $\alpha 1$  all share a similar phenotype and die soon after birth. However, it is not known whether these GFR $\alpha$  proteins can evoke intracellular signals upon the action of GDNF family proteins in the absence of Ret.

Ret and GFR $\alpha 1$  expression patterns, although similar, exhibit differences in many tissues (Trupp et al., 1997; Enomoto et al., 1998; Golden et al., 1999; Kokaia et al., 1999), which may be a sign of the distinct signaling from GFR $\alpha$  receptors alone or in conjunction with Ret tyrosine kinase *in trans* (Yu et al., 1998). We recently showed both *in vitro* and *in vivo* (Ylikoski et al., 1998), for example, that GDNF promotes survival of postnatal cochlear sensory neurons expressing GFR $\alpha 1$  mRNA but lacking Ret mRNA. This difference in expression patterns may be a sign of distinct Ret-independent signaling triggered by activation of GFR $\alpha$  receptors.

The triggering of GDNF-dependent intracellular signaling in RN33B cells has also been described (PCT/US96/18197, incorporated herein by reference). RN33B cells were described therein as expressing four putative receptors for GDNF, none of which was c-Ret. Two of the receptors were later determined to be GFR $\alpha 1$  and GFR $\alpha 2$  (reported as GDNFR $\alpha$  and GDNFR $\beta$ , respectively, U.S. Patent Application Serial No. 08/861,990, incorporated herein by reference). The mechanism of the Ret-independent signaling, however, was not known or described.

Although GPI-anchored membrane proteins have not been conclusively shown to exhibit independent intracellular signaling functions, evidence suggesting this possibility has

been increasing (Simons and Ikonen, 1997; Friedrichson and Kurzchalia, 1998; Harder et al., 1998; Varma and Mayor, 1998; Viola et al., 1999). It has been shown, for example, that GPI-anchored proteins in the immune system can mediate intracellular signaling events, such as activation of the small G-proteins, Src-type tyrosine kinases and elevation of intracellular free calcium concentration ( $[Ca^{2+}]_i$ ) (Green et al., 1997; Brown and London, 1998; Viola et al., 1999). GPI-anchored independent signaling has not previously been shown in cells of the nervous system, however.

The aim of the invention, therefore, is to further elucidate Ret independent intracellular signaling. We specifically address the role of GDNF-activated signaling in dorsal root ganglion (DRG) neurons isolated from Ret-null ( $Ret^{-/-}$ ) transgenic mice (Schuchardt et al., 1994) and in other Ret-negative cell lines, for the purpose of developing a method for identifying compounds which are agonists or antagonists of Ret independent signaling.

## 15 SUMMARY OF THE INVENTION

The present invention provides methods for screening for compounds that are agonists or antagonists of GPI-anchored receptor mediated intracellular signaling, more specifically,  $GFR\alpha 1$ -dependent, Ret-independent intracellular signaling, and methods for preventing and treating neuronal diseases comprising the use of such compounds.

20 In one aspect, the present invention relates to methods for identifying compounds which are agonists of intracellular signaling effected by GPI-anchored receptors in nervous system cells comprising incubating nervous system cells having such receptors with a test compound and determining whether intracellular signaling has been effected in the cells.

In another aspect, the present invention relates to methods for identifying compounds 25 which are antagonists of intracellular signaling effected by GPI-anchored receptors in nervous system cells comprising incubating nervous system cells having such receptors with a test compound in the presence of a sufficient amount of an agonist of such signaling, and determining whether such signaling is decreased in the cells, as compared to controls run in the absence of the compound.

In a further aspect, the present invention relates to a method for identifying compounds which are antagonists of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling by incubating cells which express GFR $\alpha$ 1 receptors, but not Ret, with a compound to be tested in the presence of a sufficient amount of an agonist of such signaling, and  
5 determining whether such signaling is decreased in the cells, as compared to controls run in the absence of the compound.

In still another aspect, the present invention relates to a method for identifying compounds which are agonists of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling by incubating cells which express GFR $\alpha$ 1 receptors, but not Ret, with a compound  
10 previously determined to bind GFR $\alpha$ 1 and determining whether the compound causes an increase in  $[Ca^{2+}]_i$ .

In a further aspect, the present invention relates to a method for identifying compounds which are antagonists of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling by incubating cells which express GFR $\alpha$ 1 receptors, but not Ret, with a compound  
15 to be tested in the presence of a sufficient amount of an agonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling effective for increasing  $[Ca^{2+}]_i$  and determining whether cells incubated with the compound have decreased  $[Ca^{2+}]_i$  levels as compared with controls not incubated with the compound.

In yet another aspect, the present invention relates to a method for identifying  
20 compounds which are agonists of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling by incubating cells which express GFR $\alpha$ 1 receptors, but not Ret, with a compound to be tested, preparing a cell lysate, immunoprecipitating the lysate with anti-GFR $\alpha$ 1 antibodies to form an immunoprecipitate, and performing assays to measure kinase phosphorylation on that immunoprecipitate.

25 In a further aspect, the present invention relates to a method for identifying compounds which are antagonists of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling by incubating cells which express GFR $\alpha$ 1 receptors, but not Ret, with a compound to be tested in the presence of a sufficient amount of an agonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling to effect said kinase phosphorylation, preparing a cell  
30 lysate, immunoprecipitating that lysate with anti-GFR $\alpha$ 1 antibodies to form an

immunoprecipitate, and performing assays to measure kinase phosphorylation on that immunoprecipitate, then comparing the results to controls run in the absence of the compound to be tested.

In yet a further aspect, the invention relates to a method for identifying agonists of  
5 GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling by incubating cells which express GPI-anchored GFR $\alpha$ 1 receptors, but not Ret, with a compound to be tested and determining whether Src-kinase is activated.

In a still further aspect, the invention relates to a method for identifying antagonists  
10 of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling by incubating cells which express GPI-anchored GFR $\alpha$ 1 receptors, but not Ret, with a compound to be tested and a sufficient amount of an agonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling to activate Src kinase and determining whether incubation with the compound has resulted in less activation of Src kinase as compared to controls not incubated with the compound.

In other aspects, the present invention relates to methods for effecting cellular  
15 responses in nervous system cells comprising administering an effective amount of either an agonist or antagonist of GPI-anchored intracellular signaling.

In yet another aspect, the present invention relates to a method for identifying  
agonists of intracellular signaling effected by GFR $\alpha$  receptors comprising incubating lipid  
rafts prepared from cells having such receptors with a test compound, and determining  
20 whether Src-type kinase is activated

In yet a further aspect, the present invention relates to a method for identifying  
antagonists of intracellular signaling effected by GFR $\alpha$  receptors comprising incubating lipid  
rafts prepared from cells having such receptors with a test compound in the presence of a  
sufficient amount of an agonist of GFR $\alpha$ -dependent signaling to activate Src-type kinase,  
25 and determining whether Src-type kinase activation is reduced in the presence of the test  
compound, as compared with controls run without the test compound.

In a further aspect, the present invention relates to methods for treating neuronal  
diseases comprising the administration of an agent which is an agonist or antagonist of GPI-  
anchored intracellular signaling.

In further aspects, the present invention relates to methods for treating neuronal diseases comprising the administration of agents which are agonists and/or antagonists of GPI-anchored intracellular signaling.

5 These and other aspects of the invention will become more apparent from the following detailed description.

### BRIEF DESCRIPTION OF THE DRAWINGS

**Figures 1 A-G** depict GDNF-evoked rapid and long-lasting  $[Ca^{2+}]_i$  changes in wild type mouse DRG neurons. The vertical bars depict  $\Delta[Ca^{2+}]_i$  changes of 100 nM. **A.** Neurons were loaded with 10  $\mu$ M Ca-Green 1AM. Increasing concentrations of 10-100 ng/ml GDNF, applied to the bath as indicated, evoked rapid and long-lasting elevations in  $[Ca^{2+}]_i$  (n=15 separate experiments, at least 3-4 neurons recorded in each experiment). **B.** 100 ng/ml GDNF, heat-inactivated at 98 °C for 15 min did not evoke changes in  $[Ca^{2+}]_i$  (8 recorded cells). In these two representative recordings thapsigargin (5  $\mu$ M) (Tha) was applied at the end of the experiments to prove the functionality of the internal calcium stores. **C.** RT-PCR shows that wild type DRG neurons express Ret (284 bp fragment), GFR $\alpha$ 1 (746 bp fragment), and GFR $\alpha$ 2 (429 bp fragment) mRNA. Neurofilament light chain (NF-L, 644 bp fragment) was used as a positive control for neuronal mRNAs. H<sub>2</sub>O lane depicts a negative control without added mRNA. The size of molecular weight markers is shown on the right. **D.** Pre-treatment with 1U/ml PI-PLC eliminates GDNF-evoked  $[Ca^{2+}]_i$  elevation. **E.** Pre-treatment with 5 $\mu$ M thapsigargin abolished the effect of GDNF. **F.** Pretreatment with the PLC $\gamma$  inhibitor U-73122 also resulted in abolishing of the effect of GDNF. **G.** GDNF (100 ng/ml) evoked both transient and sustained  $[Ca^{2+}]_i$  elevation in E18 DRG neurons isolated from GFR $\alpha$ 2-negative (GFR $\alpha$ 2<sup>-/-</sup>) mice (9 recorded neurons). In these neurons nominal removal of external Ca<sup>2+</sup> (no added EGTA, about 20  $\mu$ M free Ca<sup>2+</sup>) led to the rapid decline of  $[Ca^{2+}]_i$ . In this condition addition of GDNF evoked fast, transient and often oscillatory responses in all of these cells. Re-addition of external Ca<sup>2+</sup> (wash-out) evoked a rise in  $[Ca^{2+}]_i$  in GFR $\alpha$ 2<sup>-/-</sup> (9 recorded cells) as well as in wild type DRG neurons (8 recorded cells).



**Figures 2 A-D** depict GDNF-triggered  $\text{Ca}^{2+}$  release from the heparin-sensitive internal calcium stores in  $\text{Ret}^{-/-}$  (Ret-negative) DRG neurons. **A.** Single cell RT-PCR was performed in 18  $\text{Ret}^{-/-}$  DRG neurons. Upper panel shows  $\text{GFR}\alpha 1$  mRNA expression pattern in 11 cells. Cyclophilin (CP, lower panel) was used as a control. 16 out of 18 studied neurons expressed  $\text{GFR}\alpha 1$  mRNA. **B.** GDNF (100 ng/ml)-evoked long-lasting  $\text{Ca}^{2+}$  elevation in  $\text{Ret}^{-/-}$  DRG neurons (the two traces are representative of 41 recordings. The vertical bars depict  $d[\text{Ca}^{2+}]_i$  changes of 100 nM. **C.** Pre-treatment with PI-PLC (1 U/ml; for 1 hr at 37 °C) abolished the effect of GDNF because of removal of the GPI-anchored proteins from the membrane. To control the viability of the cells at the end of experiments the neurons were treated with thapsigargin (5  $\mu\text{M}$ ) and were also depolarized with 50 mM  $\text{K}^+$ . After thapsigargin or  $\text{K}^+$  treatment  $[\text{Ca}^{2+}]_i$  was transiently increased indicating that the internal  $\text{Ca}^{2+}$  stores and voltage-operated  $\text{Ca}^{2+}$  channels were functional. The vertical bars depict  $d[\text{Ca}^{2+}]_i$  changes of 100 nM. **D.** In two separate experiments, part of the neurons pre-loaded with Ca-green were injected with heparin. Control neurons were injected with the solvent. Heparin injected neurons (indicated with #2, lower trace,  $n=3$  neurons) did not significantly respond to GDNF application whereas control neurons responded with a long-lasting  $[\text{Ca}^{2+}]_i$  elevation which was slowly declining during wash-out (indicated with #1, upper trace,  $n=3$  neurons). Vertical bar depicts  $d[\text{Ca}^{2+}]_i$  changes of 100 nm.

**Figures 3 A-E** depict GDNF-evoked calcium entry in  $\text{Ret}^{-/-}$  DRG neurons. **A.** In part of the DRG neurons, application of nominally  $\text{Ca}^{2+}$  free extracellular solution (no added EGTA) resulted in a delayed transient  $[\text{Ca}^{2+}]_i$  elevation possibly due to activation of capacitative calcium entry (15 recorded neurons). This  $[\text{Ca}^{2+}]_i$  overshoot was not observed when calcium concentration in the nominally  $\text{Ca}^{2+}$  free external solution was clamped to about 1 nM with 2 mM EGTA (14 recorded neurons; data not shown). A return to 2 mM external  $\text{Ca}^{2+}$  (wash-out) resulted in a pronounced  $[\text{Ca}^{2+}]_i$  overshoot indicating an increased membrane permeability for  $\text{Ca}^{2+}$ . Switching back to nominally  $\text{Ca}^{2+}$  free external media in the presence of GDNF (100 ng/ml) resulted in a transient elevation of  $[\text{Ca}^{2+}]_i$  with significantly prolonged kinetics of  $[\text{Ca}^{2+}]_i$  decline. This indicates that at the resting membrane potential GDNF can prolong capacitative calcium entry either via more profound depletion of the internal stores

or by a direct action on calcium channels in the plasma membrane. The traces are representative of 14 recordings performed in 3 independent experiments. B. GDNF was repeatedly applied both in the presence of the normal extracellular  $\text{Ca}^{2+}$  concentration and in the presence of nominally  $\text{Ca}^{2+}$  free external solution in  $\text{Ret}^{-/-}$  DRG neurons. Application of GDNF (100 ng/ml) in the normal calcium extracellular solution evoked a typical long-lasting  $\text{Ca}^{2+}$  elevation which was reversed by wash-out. A switch to the nominally  $\text{Ca}^{2+}$  free external solution in the continuous presence of GDNF resulted in profound (and oscillatory in 5 out of 12 recordings) increase in  $[\text{Ca}^{2+}]_i$  followed by slow decline in  $[\text{Ca}^{2+}]_i$ . Removal of GDNF (marked with arrow 1) led to a significant decline in  $[\text{Ca}^{2+}]_i$ . Readmission of GDNF led to quick elevation in  $[\text{Ca}^{2+}]_i$  (arrows 2 and 3) (12 recorded neurons). Switching back to normal external  $\text{Ca}^{2+}$  containing solution resulted in an additional capacitative overshoot in  $[\text{Ca}^{2+}]_i$ .

**Figure 4 A-D** depict GDNF activation of  $\text{GFR}\alpha 1$ -associated Src kinases, MAP kinases and CREB in  $\text{Ret}^{-/-}$  DRG neurons. A. PP1 (10  $\mu\text{M}$ ), specific inhibitor of Src-family tyrosine kinases, reversibly blocked GDNF-evoked long-lasting  $[\text{Ca}^{2+}]_i$  elevation in  $\text{Ret}^{-/-}$  DRG neurons. The trace is representative of six recordings. B. Panel 1. Triton X-100 insoluble fraction from the lysates of GDNF (100 ng/ml, 5 min) - stimulated  $\text{Ret}^{-/-}$  mouse DRG neurons was immunoprecipitated (IP) with  $\text{GFR}\alpha 1$  antibodies. The immunoprecipitate was subjected to an *in vitro* kinase assay and revealed a major phosphorylated ~60 kD band. Panel 2. This band was not seen in the control kinase assay performed in  $\text{Ret}^{-/-}$  DRG neurons either with Protein-A Sepharose alone (a) or with bFGF antibodies instead of  $\text{GFR}\alpha 1$  antibodies (b). C. A rapid GDNF-induced phosphorylation of p42/p44 MAP kinases (upper panel) in  $\text{Ret}^{-/-}$  DRG neurons. The numbers below lanes indicate the fold induction of p42 band phosphorylation relative to control. Determination of the optical density of the bands was performed using software TINA. The lower panel shows a reprobing of the same filter with anti- $\text{GFR}\alpha 1$  mAb by Western blotting (WB) and demonstrates comparable amounts of  $\text{GFR}\alpha 1$  protein in all lanes (n=3 independent experiments). D. GDNF induced profound increase in CREB Ser-133 phosphorylation. The numbers below lanes indicate the fold induction of CREB phosphorylation relative to control. The lower panel shows the reprobing

of the same filter with anti-CREB antibodies and demonstrate comparable amount of CREB protein in all lanes.

**Figures 5 A-C** depict GDNF-stimulated Src-type kinases associated with GFR $\alpha$ 1 in the Ret negative human SHEP neuroblastoma cell line. **A.** RT-PCR analysis shows that SHEP cells express only GFR $\alpha$ 1 (538 bp fragment), but not GFR $\alpha$ 2 or Ret mRNA (expected fragments of 280 and 281 bp, correspondingly). The H<sub>2</sub>O control is as shown in Fig. 1C. **B.** Upper panel: the Triton X-100 insoluble fraction from the lysates of SHEP cells were immunoprecipitated (IP) with anti-GFR $\alpha$ 1 antibodies and assayed for *in vitro* kinase activity as described in the Materials and Methods section. The cells were non-treated (0 min) or treated with 100 ng/ml GDNF for the time indicated. The optical density of the bands was determined using a phosphoimager and a TINA program and is presented as fold increase relative to control (GDNF non-treated cells). Lower panel: the precipitates after the kinase assay were probed by Western blotting (WB) with pan-Src antibodies, which recognize Fyn, Yes and Src. **C.** Left panel (GFR $\alpha$ 1): the postnuclear lysate from SHEP cells non-treated (-) or pre-treated (+) with GDNF (100 ng/ml, 1min) was precipitated with GFR $\alpha$ 1 antibodies. Co-precipitates were assayed for kinase activity as described in Materials and Methods section. GDNF significantly increased Src type kinase activity associated with GFR $\alpha$ 1. These results are representative of 5 independent experiments. Right panel (Yes): SHEP cells were not-treated (-) or pre-treated (+) with GDNF (100 ng/ml; 1min), the Src-related kinase p62Yes was immunoprecipitated from the whole cell lysates (without preliminary IP with anti-GFR $\alpha$ 1 antibody) with an anti-Yes polyclonal antibody and assayed for kinase activity. It revealed that the major GDNF-stimulated band co-migrates with Yes type kinase of about 62 kD. The samples were normalized by protein amount.

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**Figures 6 A-C** depict GDNF-evoked phosphorylation of MAPK, CREB and ATF-1 in the Ret-negative SHEP neuroblastoma cell line. **A.** Addition of GDNF evoked transient and profound increase of p42/p44 MAPK phosphorylation (left panel). The numbers below lane indicate the fold induction of p42 phosphorylation relative to control. Lower panel shows the re-probing of the same filter with anti-GFR $\alpha$ 1 antibodies and demonstrates comparable

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amount of GFR $\alpha$ 1 protein in all lanes. The results shown are representative of four independent experiments. B. GDNF induced phosphorylation of p42/p44 MAPK was completely abolished by a 5-minute pre-treatment with PP2 even at the low concentration, 1  $\mu$ M (upper panel). The blot was re-probed with the anti-GFR $\alpha$ 1 antibodies. Lower panel shows that GFR $\alpha$ 1 protein was distributed equally in all lanes (n=2 experiments). C. GDNF treatment of SHEP cells resulted in potent induction of CREB Ser-133 phosphorylation as well as induced phosphorylation of the CREB-related protein ATF-1. The numbers below the lanes indicate the fold induction of CREB phosphorylation relative to control. The lower panel shows the re-probing of the same filter with anti-CREB antibodies (n=2 experiments).

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**Figures 7 A-C** depict GDNF-activated Src type kinase and MAP kinase in NIH 3T3 fibroblasts stably transfected with GFR $\alpha$ 1. A. The kinase assay experiments. The major ~60 kD proteins can be co-precipitated with GFR $\alpha$ 1 after GDNF (100 ng/ml) pre-treatment both in Neuro2A-20 neuroblastoma cells expressing Ret (1) and in the NIH3T3 fibroblast cells (2) (both cell lines were stably transfected with GFR $\alpha$ 1). B. GDNF induced phosphorylation of p42/p44 MAPK in NIH 3T3 cells lacking Ret (upper panel; n=3 independent experiments). Pre-treatment of the cells with selective Src kinase inhibitor, PP2 (5  $\mu$ M) for the indicated time abolished the effect of GDNF. The numbers below lanes indicate fold induction of p42 MAPK phosphorylation relative to control. The lower panel shows a re-probing of the same filter with anti-GFR $\alpha$ 1 antibodies and demonstrates comparable amounts of GFR $\alpha$ 1 protein in all lanes (n=2 independent experiments). C. Neuro2A neuroblastoma cells expressing Ret but not endogenous GFR $\alpha$ 1 was treated with GDNF in the presence of a soluble GFR $\alpha$ 1 (GFR $\alpha$ 1/Fc chimeric protein; 1  $\mu$ g/ml) lacking a GPI anchor. Soluble GFR $\alpha$ 1 induced GDNF-dependent phosphorylation of p42/p44 MAPK. The numbers below lanes indicate fold induction of p42 MAPK phosphorylation relative to control (non-treated with GDNF).

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**Figures 8 A-C** depict GDNF-increased PLC $\gamma$  tyrosine phosphorylation in SHEP neuroblastoma cells. A. SHEP cells were incubated with the indicated concentrations of GDNF for 1 min, and then lysed. Tyrosine-phosphorylated proteins were immunoprecipitated with 4G-10 anti-phosphotyrosine antibodies ( $\alpha$ -PY) and then probed for

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PLC $\gamma$  with anti-PLC $\gamma$  antibodies by Western blotting as described in the Materials and Methods. GDNF evoked a dose-dependent increase in PLC $\gamma$  tyrosine phosphorylation (n=3 experiments). The samples were normalized by protein amount. **B.** Left panel: SHEP cells were incubated with indicated concentrations of GDNF for 1 min or for 5 min. PLC $\gamma$  proteins were immunoprecipitated from the lysates by anti-PLC $\gamma$  antibodies. The immunocomplexes were probed for tyrosine-phosphorylated proteins with 4G-10 antibodies ( $\alpha$ -PY) by Western blotting. Right panel: the blot was re-probed with anti-PLC $\gamma$  antibodies. In all panels the numbers below lanes indicate fold induction of PLC $\gamma$  phosphorylation relative to control. **C.** GDNF application did not affect the phosphorylation of JNK (n=2 experiments).

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**Figures 9 A-B** depict a schematic representation of the proposed Ret-independent GDNF-evoked signaling pathway. **A.** The GDNF-triggered membrane signaling most probably occurs within lipid rafts, as GFR $\alpha$ 1 protein can be co-precipitated with Src type kinases in Triton X-100 insoluble membrane fractions. **B.** GDNF-evoked activation of GFR $\alpha$ 1 induces Src type kinase (in particular, pp62<sup>Yes</sup> kinase in SHEP cells) activation and subsequent phosphorylation of PLC $\gamma$  and MAP kinases. PLC $\gamma$  activation leads to IP $_3$ -dependent release of Ca<sup>2+</sup> from internal calcium stores. Src-dependent phosphorylation of MAPK lead to its translocation to the nucleus and CREB activation..

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## 20 DETAILED DESCRIPTION

An increasing amount of evidence suggests that GPI-linked proteins associated with lipid rafts (Sargiacomo, 1993; Fra et al., 1994; Casey, 1995; Simons & Ikonen, 1997; Xavier et al., 1998) are able to mediate intracellular signaling events *in vitro* and *in vivo* (Green et al., 1997; Simons and Ikonen, 1997; Mayor et al., 1998). The existence of microdomains of GPI-anchored proteins was recently shown in living cells (Varma & Mayor, 1998; Friedrichson & Kurzchalia, 1998). The GFR $\alpha$  proteins, as described above, are GPI-anchored.

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We disclose herein that GDNF can evoke potent intracellular signaling through a Ret-independent, GPI-anchored GFR $\alpha$ 1-mediated pathway. We also disclose that, upon GDNF binding, GPI-anchored GFR $\alpha$ 1 can solely evoke the induction of PLC $\gamma$  signaling pathway

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that is dependent on the activation of Src Family kinases and results in the long-lasting sustained  $[Ca^{2+}]_i$ . We further disclose that in addition to activating PLC $\gamma$  and calcium signaling, GDNF activates Ret-independent Src kinase-mediated ERK1/ERK2 (MAPK) and CREB in Ret<sup>-/-</sup> DRG neurons and in the different Ret-negative cell lines.

5 We further describe assays for compounds that are agonists or antagonists of GPI-anchored intracellular signaling based upon  $[Ca^{2+}]_i$  elevation and the activation of Src family kinases. We also disclose methods for identifying compounds that are agonists or antagonists of GPI-anchored intracellular signaling based upon phosphorylation of MAPK and CREB.

10 Whether a compound binds to GFR $\alpha$ 1 can be readily determined by one skilled in the art using, for example, methods for identifying compounds which bind receptors for GDNF as described in U.S. Patent Application Serial No. 08/861,990, incorporated herein by reference. Alternatively, the Biacore device (Pharmacia) can be used. Src kinase activity can be determined indirectly by looking at the tyrosine phosphorylation state or kinase  
15 activity of its substrates that include, but is not limited to, p130Cas (Sakai et al., 1997) and FAK (Polte et al., 1997). Methods for determining MAPK and CREB activation are described herein.

Activation of the GFR $\alpha$ 1-dependent, Ret-independent signaling pathways may stimulate different cellular responses than those of the Ret-dependent pathway. For instance,  
20 neuronal survival, neurite extension, enhancement of neurotransmitter synthesis, or other cellular responses to GDNF may be preferentially enhanced by one pathway over the other. Agonists or antagonists of one of these signaling mechanisms may provide for more specific cellular responses than that of GDNF itself and thereby gain therapeutic advantage over GDNF. Methods for identifying agonists and antagonists of Ret-dependent intracellular  
25 signaling is described in U.S. Patent Application Serial No. 08/861,990, incorporated herein by reference. Methods to develop agonists of both signaling pathways, antagonists of both pathways, agonists or antagonists of just one pathway, or compounds that agonize one pathway but antagonize the other pathway are contemplated herein.

GFR $\alpha$ 1-dependent, Ret-independent signaling may promote the survival and function  
30 of specific neuronal populations. Auditory neurons, which receive the impulses from the

sensory auditory hair cells and transmit them to the brain, respond to GDNF both in vitro and in vivo. GDNF has been shown to protect neurons of the inner ear. (Tay et al., 1998; Shoji et al., 1998; and Keithley et al., 1998.) GFR $\alpha$ 1 is expressed on auditory neurons in the absence of Ret. Therefore, a mechanism is described herein that suggests how the

5 Ret-independent signaling works and how this population of neurons may be supported by compounds that specifically mimic GDNF dimerization of GFR $\alpha$ 1 at the receptor or at the subsequent intracellular signaling events.

"A", "an", and "the" as used herein refer to the singular and plural.

The term "effect" as used herein means an alteration or change. An effect can be

10 positive, such as causing an increase in some material, or negative, e.g., antagonistic or inhibiting.

The term "agonist" as used herein refers to a compound or composition that can stimulate or positively influence the intracellular signaling pathways described herein, or augment or synergize the activity of any other compound or composition thereon.

15 The term "antagonist" as used herein refers to a compound or composition that can inhibit, suppress, block or negatively influence the intracellular signaling pathways described herein.

The term "sufficient amount" as used herein refers to a quantity of an agent that will result in the referred to effect.

20 The term "bind" as used herein refers to the interaction between ligands and their receptors, the binding being of a sufficient strength and for a sufficient time to allow the detection of said binding under the conditions of the assays disclosed herein.

The term "about" in reference to a numerical value means  $\pm 10\%$  of the numerical value, more preferably  $\pm 5\%$ , most preferably  $\pm 2\%$ .

25 The term "administration" includes but is not limited to, oral, subbuccal, transdermal, parenteral, subcutaneous and topical. A common requirement for these routes of administration is efficient and easy delivery.

As used herein, the term "effective amount," refers to the amount required to achieve an intended purpose for both prophylaxis or treatment without undesirable side effects, such

30 as toxicity, irritation or allergic response. Although individual needs may vary, the

determination of optimal ranges for effective amounts of formulations is within the skill of the art. Human doses can readily be extrapolated from animal studies (Katocs *et al.*, Chapter 27 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990). Generally, the dosage required to provide an effective amount of a  
5 formulation, which can be adjusted by one skilled in the art, will vary depending on several factors, including the age, health, physical condition, weight, type and extent of the disease or disorder of the recipient, frequency of treatment, the nature of concurrent therapy, if required, and the nature and scope of the desired effect(s) (Nies *et al.*, Chapter 3 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman *et al.*,  
10 eds., McGraw-Hill, New York, NY, 1996).

The term "nervous system cell" as used herein refers to all cells present in or derived from the nervous system, including, but not limited to neuronal cells, such as neurons, and non-neuronal cells, such as glial cells.

The term "transformed cell" as used herein refers to a cell that has been modified  
15 using procedures known in the art to express GFR $\alpha$ 1 and/or not to express Ret.

The term "neuronal disease," as used herein, means any disturbance in structure or function of any nervous system cells, from whatever cause, and shall include all abnormalities, whether originating genetically or environmentally, present congenitally or later acquired, and from any cause, whether infectious, traumatic, toxic, degenerative,  
20 inflammatory or neoplastic. This shall include any neurodegenerative or retrogressive process within one or more cells of the nervous system, including even the death of nerves, axons, or tracts of the central nervous system.

The term "cellular response" as used herein refers to, without limitation, any change in neuronal survival, neuronal plasticity, neurite extension, cell migration, or any  
25 enhancement or inhibition of neurotransmitter synthesis and/or release.

The term "lipid rafts," as used herein, refers to a structure of sphingolipids and cholesterol packed into moving platforms within the liquid bilayer of cell membranes, and includes the detergent insoluble, glycolipid-enriched fraction that remains after extraction with Triton X-100 or similar detergents.



The terms "GPI-anchored" or "GPI-linked" as used herein in reference to a receptor refer to a receptor that is associated with GPI.

The term "independent intracellular signaling" in reference to a receptor as used herein refers to a receptor that evokes intracellular signaling without requiring and/or in the  
5 absence of co-receptors.

We surprisingly found that long-lasting  $[Ca^{2+}]_i$  elevations were obtained in response to GDNF (10-100 ng/ml) in DRG neurons isolated from the  $Ret^{-/-}$  mice. Investigation of this phenomena showed that GDNF-evoked  $[Ca^{2+}]_i$  elevation persisted in nominally free extracellular  $Ca^{2+}$  solution, and can be effectively blocked by either removing GPI-linked  
10 proteins, PLC $\gamma$  inhibition or by the intracellular administration of low molecular weight heparin, an antagonist of  $IP_3$ -sensitive  $Ca^{2+}$  release channels on internal stores. Thus in  $Ret^{-/-}$  DRG neurons GDNF triggers the release of  $Ca^{2+}$  from the internal  $Ca^{2+}$  stores perhaps via the PLC $\gamma$ -dependent  $Ca^{2+}$  release pathway. Presently we cannot, however, exclude the existence of another calcium release mechanism. Unfortunately, U-73122 is not a selective inhibitor of  
15 only PLC $\gamma$  and heparin may also have other effects that of a competitive block of the  $IP_3$  binding sites at calcium release channels. Nevertheless, both low molecular weight heparin used in this study (Thorn et al., 1993; Meyer zu Heringdorf, 1998; Takei et al., 1998 and ref. thereafter) and U-73122 (Bourette et al., 1997; Davletov et al., 1998; Stam et al., 1998) are the most widely used pharmacological tools for complex investigation of PLC $\gamma$  dependent  
20 signaling.

In the later phase of Ret-independent long-lasting  $Ca^{2+}$  elevation, the calcium entry through calcium release activated channels (CRAC) would play a role in the maintenance of the elevated  $[Ca^{2+}]_i$  due to balance refilling of intracellular  $Ca^{2+}$  stores (Sharenberg and Kinet, 1998). Sustained elevations in  $[Ca^{2+}]_i$  may lead to long term potentiation of various cellular  
25 responses, including, without limitation, major alterations in gene expression, neuronal plasticity, neurite extension, and neuron survivability, and may play an important role in such areas as learning ability, memory, epilepsy, and hearing loss, to name but a few. Potent capacitative calcium entry aimed in re-filling of depleted calcium stores exists in DRG neurons (Usachev et al., 1999).

We disclose that GDNF can stimulate a different pattern of  $[Ca^{2+}]_i$  elevation in Ret-containing wild type and  $GFR\alpha 2^{-/-}$  DRG neurons via the triggering of calcium release from heparin and PLC $\gamma$ -sensitive internal stores. Ret tyrosine kinase has been shown to evoke a potent PLC $\gamma$  activation through a PLC $\gamma$  binding site (Borrello et al., 1996). Although there is  
5 a common view that *in vivo* GDNF preferentially binds to  $GFR\alpha 1$  and NTN binds to  $GFR\alpha 2$ , GDNF at high concentrations *in vitro* can activate  $GFR\alpha 2$  and NTN can bind to  $GFR\alpha 1$  (Buj-Bello, 1997; Jing et al., 1997; Klein et al., 1997; Suvanto et al., 1997; reviewed by Airaksinen et al., 1999). To study the contribution of  $GFR\alpha 2$  in the action of GDNF we investigated the effect of GDNF on  $[Ca^{2+}]_i$  in  $GFR\alpha 2$ -negative ( $GFR\alpha 2^{-/-}$ ) DRG neurons  
10 isolated from  $GFR\alpha 2$  deficient mice (Rossi et al., 1999). We found that the GDNF-dependent  $[Ca^{2+}]_i$  rise in DRG neurons was specifically dependent on  $GFR\alpha 1$  activation, since GDNF evoked the same pattern of  $[Ca^{2+}]_i$  elevation in  $GFR\alpha 2^{-/-}$  neurons as in the wild type DRG neurons.

We also disclose that the Ret-independent GDNF-evoked intracellular signaling in  
15 neurons is much broader than activation of different patterns of  $Ca^{2+}$  elevation. In addition to the GDNF-evoked  $Ca^{2+}$  rise we detected a potent GDNF-dependent transient activation of MAPK and CREB in Ret $^{-/-}$  DRG neurons. GPI-anchored protein-coupled kinases and Src-type kinases in particular, have been shown to evoke PLC $\gamma$  stimulation,  $[Ca^{2+}]_i$  elevation and MAPK activation (Brown and London, 1998; Dikic et al., 1996; Finkbeiner and Greenberg,  
20 1998; Khare et al., 1997; Luttrell et al., 1996, 1997; Thomas and Brugge, 1997). Indeed in our experiments low doses of (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1) or (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) (both from Calbiochem), the potent and specific inhibitors of Src-type kinases, reversibly blocked GDNF-evoked  $[Ca^{2+}]_i$  elevation in the Ret-negative DRG neurons as well  
25 as inhibited GDNF-dependent activation of MAPK in Ret-negative cell lines. Thus we wanted to investigate how GPI-anchored  $GFR\alpha 1$  proteins lacking of an intracellular domain can, nevertheless, couple to and activate Src and MAP kinases at the cytoplasmic side of the membrane. To explore this question we used Ret-negative cell lines, SHEP neuroblastoma cells and NIH3T3 fibroblasts stably transfected with  $GFR\alpha 1$  cDNA.

According to current understanding (Simons and Ikonen, 1997; Brown and London, 1998) GPI-anchored proteins, transmembrane tyrosine kinase proteins, G-proteins and acylated tyrosine kinases of the Src family all can associate with so-called lipid rafts, a structure of sphingolipids and cholesterol packed into moving platforms within the liquid bilayer (Sargiacomo et al., 1993; Simons and Ikonen, 1997; Brown and London, 1998; 5 Luttrell et al., 1999; Viola et al., 1999). Whether or not such lipid rafts exist in DRG neurons is not known. It is also not known whether GDNF family receptors are included in the rafts. An association of Src type kinases with the inner leaflet of the raft by myristilation or palmytilation links has already been demonstrated for many cell types, however (Brown 10 and London, 1998).

To test whether GFR $\alpha$ 1 can be coupled with a Src-type kinase within a raft, we performed experiments involving co-precipitation of kinases possibly coupled to the GFR $\alpha$ 1 protein using GFR $\alpha$ 1 antibodies in Triton X-100 detergent resistant membrane fractions. After Triton X-100 extraction, insoluble lipids and proteins remain in the form of detergent- 15 insoluble glycolipid-enriched complexes(DIGs) or lipid rafts (reviewed by Simons and Ikonen, 1997). We disclose herein that Src-type kinases can be co-precipitated with GFR $\alpha$ 1 in DIGs from Ret<sup>-/-</sup> DRG neurons as well as in the different Ret-negative but GFR $\alpha$ 1-expressing cell lines. In DIGs from SHEP neuroblastoma cells, GDNF evoked a potent transient activation of Src kinase. These findings indicate that activation of Src-type kinases 20 by GDNF in Ret-negative DRG neurons and neuroblastoma cells might occur within the lipid rafts.

As in the wild type DRG neurons, GDNF evoked potent activation of p42/p44 MAPK and CREB in the different Ret-deficient cell lines. Again GDNF-evoked phosphorylation of MAPK was completely abolished with low doses of the selective Src 25 kinase inhibitor, PP2. By immunostaining with anti-phosphorylated MAPK antibodies we also found that phosphorylated MAPK is effectively translocated to the nucleus of SHEP cells and this translocation was modulated by PP2 (Poteriaev and Titievsky, unpublished observations). Thus GDNF-evoked Ret-independent phosphorylation of MAPK is completely dependent on the GDNF-evoked activation of a Src type kinase. Since the 30 studies on neuronal survival showed that MAPK effectively phosphorylates CREB, it was of

great interest to reveal the downstream targets of activated MAPK in Ret-negative neurons and cell lines. Indeed, we detected significant GDNF-dependent activation of CREB both in the Ret<sup>-/-</sup> DRG neurons and the SHEP cells. Our results are therefore to some extent contradictory to those of Trupp et al. (1999), which found that GDNF Ret-independently  
5 activates CREB phosphorylation but not Ras/ERK pathway in RN33B cells. In addition to the detection of potent GDNF-dependent ERK1/ERK2 activation, we observed a significantly prolonged kinetic of CREB activation in SHEP cells as compare to the Trupp et al. (1999) study conducted in RN33B cells. Interestingly, we also observe a robust GDNF-dependent activation of the CREB-related protein ATF-1 in Ret-negative SHEP cells but not  
10 in the Ret<sup>-/-</sup> DRG neurons. It is, therefore, possible that the noticed discrepancies between our results and those reported by Trupp et al. (1999) reflect the variance between the different type of the cells.

Src kinases and MAPK might significantly affect cell function since these kinases have been established to be crucially involved in mitogenesis, nerve-growth factor induced  
15 cell differentiation with neurite outgrowth, cell migration as well as in focal adhesion kinase (FAK) dependent cell motility (Khare et al., 1997; Thomas and Brugge, 1997). A potent MAPK activation, such as observed in our experiments, might promote neuronal survival and neuronal plasticity (reviewed by Fukunaga K. & Miyamoto E., 1998 and Impey et al., 1999). At the moment, however, we do not know the exact physiological meaning of the  
20 GDNF invoked long-lasting Ca<sup>2+</sup> elevation and MAPK and CREB activation in Ret<sup>-/-</sup> DRG neurons and in the cell lines. Ret-independent GDNF-evoked activation of the transcription factors of the CREB family can lead to a potent up-regulation of the gene's expression. This can result in the profound changes in neuronal plasticity (Finkbeiner et al., 1997), since it has been shown that MAPK signaling facilitates memory consolidation and long-term  
25 potentiation by promoting de novo CREB-regulated gene expression (reviewed by Impey et al., 1999). Unfortunately, the study of the possible changes in neuronal plasticity invoked by GDNF in Ret<sup>-/-</sup> or GFR $\alpha$ 1 deficient neurons is precluded by the early postnatal death of the knock-out animals. It would appear that DRG neurons do not use this GFR $\alpha$ 1-mediated signaling as a survival factor, as GDNF has also not been shown to stimulate survival of Ret<sup>-/-</sup>  
30 DRG neurons in culture (unpublished observations and Taraviras et al., 1999). We recently

showed, however, that GDNF promotes survival of postnatal cochlear sensory neurons, which express GFR $\alpha$ 1 mRNA but lack Ret mRNA (Ylikoski et al., 1998). The morphological and biological consequences which might be triggered by GDNF-evoked Ret-independent signaling remain to be elucidated, but it is clear that different neuronal

5 populations use this signaling pathway for different purposes and with different results.

One question that remains is how a signal is passed from the outer leaflet of the raft to the inner one, or how it is passed from the outside part of the membrane to the inner one? Without intending to be limited by any theory or mechanism, we propose that there may be an unknown transmembrane protein linking GFR $\alpha$ 1 and Src-type protein kinase that serves

10 as the transducer of the signal. One such adapter plasma membrane-associated protein has been found recently in DRG neurons (Lang et al., 1998). In our experiments an about 72 kD unrecognised protein was immunoprecipitated with GFR $\alpha$ 1 antibodies. However, the interaction of a potential adapter protein with GFR $\alpha$ 1 should be different from the Ret-GFR $\alpha$ 1 coupling. The soluble GFR $\alpha$ 1, capable of inducing MAPK phosphorylation in the

15 presence of GDNF in Ret-expressed cells, was unable to evoke intracellular signaling in the Ret-negative parental NIH3T3 fibroblasts. Based on these experiments we conclude that if an adapter protein exists, it would, unlike Ret, strictly require an association with the GPI anchor of GFR $\alpha$ 1. Another possibility may be that enzymes activated in the rafts involved in anchor release might yield soluble phospho-oligosaccharides. These would then flip across

20 the bilayer and may function as active second messengers in the cytosol. GDNF can also trigger lipid-lipid interaction and raft coalescence-dependent accumulation of intracellular phosphorylated proteins. Whether such signaling pathways are involved in the action of GDNF is presently unknown.

In summary, we have found that GDNF evokes [Ca<sup>2+</sup>]<sub>i</sub> elevation and Ret-

25 independent activation of Src-tyrosine kinase, PLC $\gamma$ , MAPK and CREB - coupled intracellular signaling pathways in Ret<sup>-/-</sup> DRG neurons and in the Ret-negative cell lines. GDNF binds to the GPI-anchored GFR $\alpha$ 1 with subsequent activation of Src kinases associated with GFR $\alpha$ 1 followed by activation of MAPK, CREB, PLC $\gamma$  and sustained elevation of [Ca<sup>2+</sup>]<sub>i</sub>. The proposed signaling pathway is summarized in Fig. 9.

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## EXAMPLES

### Materials and methods

#### *Neuronal cultures*

5           DRG from embryonic day 18 (E18) mice were treated with trypsin (Worthington), non-neuronal cells removed by preplating, and about 95% pure neurons cultured on poly-ornithine-laminin-coated glass coverslips in Ham's F14 medium (Imperial Laboratories) with serum substitute containing NGF, BDNF and NT-3 (all from PeproTech, 2 ng/ml each) for at least 1 day before measurement. Neurotrophins were extensively washed out  
10 before GDNF application. GDNF was from PeproTech and donated by Cephalon, Inc. Ret-deficient mice (Schuchardt et al., 1994) were identified from heterozygote matings by the absence of kidneys and PCR-based genotyping. GFR $\alpha$ 2-deficient mice were obtained from homozygote matings (Rossi et al., 1999).

#### *[Ca<sup>2+</sup>]<sub>i</sub> measurement*

15           [Ca<sup>2+</sup>]<sub>i</sub> was measured using a Bio-Rad MRC-1024 confocal microscope equipped with an argon-krypton laser. The cells were loaded with 10  $\mu$ M Calcium Green-1AM (Molecular Probes), a membrane permeable Ca<sup>2+</sup> dye, by 30 min incubation in serum free cell-culture media at 37 °C and 5 % CO<sub>2</sub>. After loading, the neurons were kept at room temperature in Dulbecco's PBS solution contained 20 mM HEPES (pH = 7.4) for at least  
20 20 min prior to the experiments. All compounds were applied to the bath by a peristaltic pump. 6 kD Heparin (Sigma) and Alexa 568 nm fluorescent dye (Molecular Probes) were loaded into the cells using Molecular Probes' pinocytotic influx loading reagent basically according to the manufacturer instructions. The loading did not affect neuronal viability throughout the experiments. Cell injection was performed using Eppendorf microinjection  
25 system. The average intensity of fluorescence in the pre-defined ROI (Region Of Interest) was measured on-line using TimeCourse software from BioRad. The calibration of fluorescence traces was performed *in vitro* using F<sub>max</sub> and F<sub>min</sub> values obtained with the calcium calibration buffer kit (Molecular Probes). Because the calibration solutions may not reflect the intracellular environment, the experimental data are presented as delta  
30 [Ca<sup>2+</sup>]<sub>i</sub> calculated using K<sub>d</sub>=190 nM for Calcium Green-1.

### *Transgenic GFR $\alpha$ 2 mice*

Transgenic GFR $\alpha$ 2 knock-out mice were produced in our laboratory. To isolate GFR $\alpha$ 2 genomic clones, we screened a mouse 129/Sv library (Stratagene) with a rat GFR $\alpha$ 2 cDNA fragment as a probe. A 6.7 kb HindIII-XbaI fragment was used to construct the targeting vector. A 0.5 kb NotI-XbaI fragment of the GFR $\alpha$ 2 gene, containing part of the first coding exon with the translation initiation site, was replaced with a 2.0 kb cassette containing the neomycin-resistance gene (neo) driven by the PGK promoter and polyadenylation signal. R1 embryonic stem cells were electroporated with linearized plasmid and selected in G418 (250ug/ml). Resistant clones were screened by Southern blot analysis using a 5' outside probe that recognizes a 7.8 kb wild-type and 5.5 kb mutant band after a BamHI digest. Positive clones were further hybridized with neo and 3' outside probes to exclude random integration of the vector. Two injected clones gave germline transmission, when the chimeras were crossed to C57BL/6J OlaHsd.

### *Single cell RT-PCR*

A negative pressure was applied to the micropipette, and the whole cell was harvested under visual control. The content of the pipette was expelled into a test tube containing Trizol™ reagent lysis buffer (Gibco BRL) and 1 µg of carrier tRNA. The total RNA was isolated and the RT-PCR reaction was performed using a Titan™ One Tube RT-PCR kit (Boehringer Mannheim) according to the manufacturer's instructions. One half of the material from each neuron was used to amplify GFR $\alpha$ 1 mRNA. The presence of total RNA in a sample was ensured by the amplification of a 216 bp fragment of cyclophilin mRNA using the second half of the material. For cyclophilin amplification, the primers from a QuantumRNA™ kit (Ambion, TX) were used. The primers used for analysis of GFR $\alpha$ 1 expression were as follows: 5'-GCGGCACCATGTTCTAGCC-3' (SEQ ID NO: 1) and 5'-CAGACTCAGGCAGTTGGGCC-3' (SEQ ID NO: 2). The primers were designed to cross at least one intron and to exclude amplification from genomic DNA. Amplification was carried out for 45 cycles at 95°C for 45 s; 62°C for 45 s; and 72°C for 60 s. The resulting fragments were identified by Southern blotting with a <sup>32</sup>P-labelled cDNA insert of the mouse GFR $\alpha$ 1 clone and a cDNA fragment of cyclophilin. Radioactive

signals were detected with a Fuji Bioimage analyzer BAS 2000. No fragments were obtained from the media of cultured neurons.

***RT-PCR analysis of GDNF receptors in cultured DRG neurons and SHEP neuroblastoma cell line.***

- 5           Total RNA from cultured DRG neurons (approximately 200 cells) was divided into three equal parts and the RT-PCR was carried out as above. The primers for GFR $\alpha$ 2 were: 5'-TATTGGAGCATCCATCTGGG-3' (SEQ ID NO: 3) and 5'-AGCAGTTGGGCTTCTCCTTG-3' (SEQ ID NO: 4), and for Ret they were: 5'-ATGAAAGGGTACTGACCATGG-3' (SEQ ID NO: 5) and 5'-AGGACCACACATCACTTTGAG-3' (SEQ ID NO: 6). The PCR was carried out for 40 cycles under the conditions indicated above. In the RT-PCR analysis of SHEP cells we used the primers for human GFR $\alpha$ 1, GFR $\alpha$ 2 and Ret. The primers sequences and PCR conditions were reported by Hishiki et al. (1998).

***Immuno-complex kinase assays and immunoblotting***

- 15           DRG neurons ( $5 \times 10^5$ ), SHEP neuroblastoma cells ( $10^7$ ), Neuro2A neuroblastoma cells stably transfected with GFR $\alpha$ 1 ( $10^7$ ) or NIH3T3 cells (NIH3T3/pBpGFR $\alpha$ 1) stably transfected with GFR $\alpha$ 1 ( $10^7$ ) were incubated in serum-free culture medium without (control) or with GDNF (GDNF-treated) for 0.5-15 min at 37°C. The cells were washed twice with cold PBS/vanadate and lysed in TX-100 lysis buffer (50 mM Tris-HCl, pH 7.5, 20 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors (Boehringer) on ice for 1 hour. The postnuclear lysates were pre-cleared by incubation with 50  $\mu$ l of 50% Protein G-Sepharose for goat polyclonal antibodies or Protein A-Sepharose CL4B (Pharmacia) for rabbit polyclonal antibodies for 1 hour at +4°C. After removal of the beads, the supernatants were incubated with 0.4-0.5  $\mu$ g of anti-GFR $\alpha$ 1 or anti-Yes polyclonal 25 antibodies (Santa Cruz Biotechnology) overnight at +4°C followed by incubation with Protein G or A-Sepharose for 2 hours. The immunocomplexes were washed twice in TX-100 lysis buffer without EDTA and twice in kinase buffer (25 mM Hepes, pH 7.4, 5 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 1 mM sodium orthovanadate). Immunocomplexes were incubated in 30 a kinase buffer supplemented with 5-10  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP for 20 minutes at 37°C. The



samples were washed out from incorporated label and were subjected to 10% SDS-PAGE. Proteins were transferred to a Hybond ECL membrane (Amersham) using semi-dry blot apparatus (Schleicher & Schuell, Dassel, FRG). Labeled proteins were visualized with a Fuji Bioimage analyzer BAS 2000 or by autoradiography. Quantification of the optical density of the blots was performed using the TINA program.

For immunoblotting, the membranes were probed with Src-2 antibodies (Santa Cruz Biotechnology) recognizing c-Src, Fyn and Yes, followed by the secondary HRP-conjugated anti-rabbit antibodies (Sigma). The membranes were developed with ECL reagents (Amersham Life Science).

#### 10 *Immunoprecipitation and Western blotting of PLC $\gamma$*

To determine changes in GDNF-evoked PLC $\gamma$  tyrosine phosphorylation the Western blotting assays were performed as described in Khare et al. (1997). Briefly, SHEP neuroblastoma cells were pre-incubated with 1 mM sodium vanadate in serum-free medium for 30 min. at 37 °C. and then were treated at 37 °C with 1-100 ng/ml GDNF for 15 min. unless otherwise indicated. Incubation was stopped by the addition of ice-cold PBS buffer containing 1 mM sodium vanadate. Whole cell lysates (final protein concentration 0.1-0.3 mg/ml) were prepared at 4 °C in an extraction buffer (pH 7.5) containing 50 mM Tris-HCl, 137 mM NaCl, 1 mM sodium vanadate, 1 mM PMSF, 10% glycerol, 1% NP-40 and protease inhibitors cocktail (Boehringer). Phosphotyrosine-containing proteins were immunoprecipitated with 4G-10 antibodies (Upstate Biotechnology), collected with Protein A Sepharose beads and separated on the 7.5% SDS-PAGE. The proteins were transferred to Hybond-ECL nitrocellulose membrane, and PLC $\gamma$  was assessed by Western blotting using anti-PLC $\gamma$  antibodies (Upstate Biotechnology). In some experiments we first immunoprecipitated PLC $\gamma$  from the cell lysate using anti-PLC $\gamma$  antibodies and probed the 25 filter with anti-phosphotyrosine 4G-10 antibodies as described above. In these experiments after the phosphotyrosine detection of the immunoprecipitates, the membranes were stripped and re-probed with anti-PLC $\gamma$  antibodies (Upstate Biotechnology). Membranes were developed with ECL reagents.

**MAPK, JNK and CREB phosphorylation assays**

To assess GDNF-dependent MAPK and CREB phosphorylation in all studied cell lines, the semiconfluent cell monolayers were starved for 3 hours in serum-free medium, and then GDNF was applied for the indicated time. For the analysis of MAPK and CREB activation in *Ret*<sup>-/-</sup> animals the DRG neurons were dissected from E18 mice and maintained in NGF-containing medium for 2 hours. After this time the neurons were deprived of NGF by placing them in NGF-free medium in the presence of anti-NGF antibodies. After 2 hr without NGF, the neurons were stimulated with GDNF. In the experiments involving Src-kinase inhibition, a Src-family kinase inhibitor PP2 was added at the indicated concentrations to the cell monolayers 5 min before GDNF application. In some experiments 1 µg/ml of soluble GFRα1 lacking a GPI anchor (GFRα1/Fc chimeric protein; R&D systems) was added 5 min prior to the GDNF application and was kept in the solution during the GDNF treatment. After stimulation, the cells were briefly washed with PBS/sodium vanadate and lysed in the buffer containing TBS, 2 mM EDTA, 1% NP-40, 1% Triton X-100, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub> and a Complete™ protease inhibitors cocktail (Boehringer Mannheim). Total cell protein for each extract was measured by MicroBSA (Pierce) and an equivalent amount of protein was resolved electrophoretically on 10% polyacrylamide gels. The proteins were transferred to a Hybond ECL (Amersham) membrane and the blot was probed with either MAPK (ERK1/2) or JNK Anti-Active™ pAbs (Promega) according to the manufacturer's instructions. The blots were then re-probed with GFRα1 mAbs (Transduction Laboratories). In the CREB phosphorylation study, Western blots were probed with the antibodies that specifically recognise the Ser-133 phosphorylated form of CREB and then re-probed with the anti-CREB antibodies (New England Biolabs).

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**Example 1*****Effect of GDNF on [Ca<sup>2+</sup>]<sub>i</sub> in Ret / GFRα1 positive wild type and GFRα2<sup>-/-</sup> DRG neurons***

In order to investigate a possible Ret-independent GDNF signaling in neurons we followed GDNF-induced changes in intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) by confocal

microscopy in Ret-expressing and Ret<sup>-/-</sup> DRG neurons. Two different types of GDNF-evoked changes in  $[Ca^{2+}]_i$  were observed in 60-70% of the wild type DRG neurons expressing both Ret, GFR $\alpha$ 1 and GFR $\alpha$ 2 (Fig. 1C). GDNF (10 ng/ml) induced a rapid transient increase in  $[Ca^{2+}]_i$ , whereas the concentrations over 10 ng/ml (10-100 ng/ml) evoked both a transient and slow long-lasting elevation in  $[Ca^{2+}]_i$  (Fig. 1A) (n=15 experiments; at least 3-4 neurons were recorded in each experiment). To control the specificity of the observed GDNF-evoked long-lasting  $Ca^{2+}$  elevation, we used heat-inactivated GDNF. Heat-inactivated GDNF did not evoke any changes of basal  $[Ca^{2+}]_i$  (8 recorded cells) (Fig. 1B). To monitor the functionality of the internal calcium pools at the end of the experiments we usually applied 5  $\mu$ M thapsigargin, an inhibitor of SERCA pump at intracellular calcium stores. All cells responded to the application of thapsigargin with a profound elevation in  $[Ca^{2+}]_i$  (two representative recordings shown on Fig. 1B). Further control experiments were performed using cleavage of GPI-anchored proteins from the membrane with phosphatidylinositol-specific phospholipase C (PI-PLC) (7 recordings). None of the pre-treated neurons responded to the application of GDNF (100 ng/ml) (Fig. 1D). Depletion of intracellular  $Ca^{2+}$  stores with 5  $\mu$ M thapsigargin (5 recordings) or pre-treatment of neurons with the PLC $\gamma$  inhibitor, U-73122 (6 recordings) also resulted in the abolishing of the effect of GDNF (Figs. 1E and 1F respectively). Consistent with the specificity of GDNF (Rossi et al., 1999) responded to GDNF with the same pattern of fast and slow kinetics of  $Ca^{2+}$  elevation as the wild type neurons (Fig. 1G) (9 recordings). Nominal removal of extracellular calcium (with no added EDTA to the extracellular media  $[Ca^{2+}]_o$  was about 20  $\mu$ M) led to decline in  $[Ca^{2+}]_i$ . Interestingly, application of GDNF in these cells resulted in a profound rapid and oscillatory (5 out of 9 cells)  $[Ca^{2+}]_i$  increase (Fig. 1D). Switching the perfusion back to the calcium-containing medium resulted in a profound calcium overshoot. Our results are consistent with a recent report, showing that depletion of intracellular calcium stores in DRG neurons evoke a substantial calcium entry aiming to re-fill these stores (Usachev et al., 1999).

**Example 2*****Effect of GDNF on  $[Ca^{2+}]_i$  in  $Ret^{-/-}$ ,  $GFR\alpha 1$  positive DRG neurons***

Ret tyrosine kinase can activate cytoplasmic PLC $\gamma$  through the PLC $\gamma$  docking site (Borrello et al., 1996), and this could lead to IP $_3$  production and subsequent Ca $^{2+}$  release.

5 We therefore assessed the role of Ret in the GDNF-evoked calcium signaling using DRG neurons, isolated from  $Ret^{-/-}$  mice (Schuchardt et al., 1994). In 16 out of 18 tested  $Ret^{-/-}$  DRG neurons we detected  $GFR\alpha 1$  mRNA (11 representative lanes shown on Fig. 2A) meaning, in agreement with a recently published observation (Natarajan et al., 1999), that  $Ret^{-/-}$  neurons preserved the expression of  $GFR\alpha 1$  mRNA well. Surprisingly, although we

10 never observed a GDNF-dependent fast  $[Ca^{2+}]_i$  rise in these neurons, a long-lasting  $[Ca^{2+}]_i$  elevation was recorded in response to 10-100 ng/ml GDNF in 60-70% of the neurons (Fig. 2B) (n=21 experiments, 41 out of 61 neurons responded). In the control experiments DRG neurons did not respond with  $[Ca^{2+}]_i$  elevations to changes of the superfusion solutions, brief switch on/off of the peristaltic pump, or prolonged application of heat-inactivated

15 GDNF (100 ng/ml) (3 experiments, data not shown; the experimental conditions were identical to experiments with wild type DRG neurons, see Fig. 1B). In addition the prolonged application of GDNF did not affect  $[Ca^{2+}]_i$  in  $Ret^{-/-}$  neurons pre-treated with PI-PLC (Fig. 2C, 3 recordings). As in the experiments with wild type DRG neurons, the viability of the neurons was verified either by depletion of intracellular stores with

20 thapsigargin (5  $\mu$ M) or by neuronal depolarization evoked by 50 mM external K $^{+}$  (Fig. 2C).

**Example 3*****GDNF triggers Ca $^{2+}$  release from the internal stores in  $Ret$  negative DRG neurons***

25 Activation of PLC $\gamma$  apparently leads to IP $_3$  production and subsequent Ca $^{2+}$  release from IP $_3$  sensitive stores. To block IP $_3$  receptors on internal Ca $^{2+}$  stores, we either injected DRG neurons with an intracellular-like solution containing 6kD heparin, a competitive antagonist of the IP $_3$  binding sites at the IP $_3$ -sensitive Ca $^{2+}$  release channels (Berridge, 1998) or loaded heparin into the cells using a pinocytotic loading reagent in a mixture with

30 Alexa 568 fluorescent dye (n=2 experiments). In the heparin injected neurons, GDNF did

not evoke an increase in  $[Ca^{2+}]_i$  in comparison to the saline injected control neurons (Fig. 2D). Neurons loaded by pinocytosis with heparin and the Alexa dye replied with  $[Ca^{2+}]_i$  elevation in response to both thapsigargin and to high extracellular  $K^+$ -evoked depolarization. They, however, did not respond either to 100 nM GDNF or to 10  $\mu$ M ATP (data not shown). The GDNF-evoked long-lasting  $[Ca^{2+}]_i$  elevation was completely abolished by pre-treatment of  $Ret^{-/-}$  neurons with U-73122, an inhibitor of PLC $\gamma$  (n=3 experiments, data not shown). Taken together, these results indicate that in  $Ret$ -negative DRG neurons GDNF can trigger  $Ca^{2+}$  release from  $IP_3$ -sensitive internal calcium stores via the  $GFR\alpha 1/PLC\gamma$  coupled pathway.

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#### Example 4

##### *Effect of GDNF on calcium entry*

We hypothesized that in addition to the GDNF-evoked release of sequestered  $Ca^{2+}$ , entry of extracellular  $Ca^{2+}$  would participate in the maintenance of the elevated  $Ca^{2+}$  level observed after stimulation with GDNF. Furthermore, it has recently been shown that depletion of intracellular calcium stores in DRG neurons results in a substantial capacitative calcium entry (Usachev et al., 1999). In 15 out of 24  $Ret^{-/-}$  neurons the switch to nominally  $Ca^{2+}$  free external solution ( $[Ca^{2+}]_o$  about 20  $\mu$ M  $Ca^{2+}$ ; the gradient of calcium over the plasma membrane was still about 200-fold under these conditions) resulted in pronounced  $Ca^{2+}$  overshoot (Fig. 3A). Since this overshoot was never observed in the cells bathed in an external solution containing 2 mM EGTA ( $[Ca^{2+}]_o$  about 1 nM; n=14 recordings, data not shown) we suggest that the perfusion of some cells with nominally calcium free solution resulted in a rapid depletion of very labile calcium pools and concomitant capacitative entry of calcium. The refilling of intracellular stores at 20  $\mu$ M external calcium was not complete, since re-addition of normal (2 mM calcium containing) extracellular solution resulted in an additional large calcium overshoot. Re-application of GDNF in the presence of low extracellular calcium (Fig. 3A) (traces are representative of 15 recordings) evoked a massive  $[Ca^{2+}]_i$  overshoot and substantially slower decay kinetics, compared with control application of nominally calcium free extracellular solution only (Fig. 3A). We concluded that GDNF prolonged capacitative calcium entry either via

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additional depletion of intracellular stores or via a direct effect on the plasma membrane calcium release activated channels (CRAC). In another set of experiments we tested if the prolonged decay of calcium entry is dependent on the direct action of GDNF on CRAC. In these experiments we removed GDNF from the nominally calcium free extracellular media at the middle of calcium overshoot. Representative traces of 12 recordings performed in *Ret*<sup>-/-</sup> DRG neurons in three separate experiments are shown on Fig. 3B. Application of GDNF (100 ng/ml) in normal calcium-contained extracellular solution resulted in the usual long-lasting  $[Ca^{2+}]_i$  elevation interrupted with a wash-out period. Switching to nominally calcium free (about 20  $\mu$ M free calcium-containing) extracellular solution resulted in a large calcium overshoot, similar to that shown in Fig. 3A. During the  $[Ca^{2+}]_i$  decay GDNF was removed from the external media (indicated on Fig. 3B with arrow 1). This resulted in rapid decline of  $[Ca^{2+}]_i$  suggesting that GDNF may have a direct regulatory effect on the plasma membrane CRAC, rather than an indirect effect mediated via depletion of calcium from internal stores. Re-addition of GDNF (100 ng/ml; indicated with arrows 2 and 3) in the nominally free calcium solution resulted in a profound increase in  $[Ca^{2+}]_i$  (Fig. 3B; 12 recorded neurons).

### Example 5

#### ***GDNF activates a GFR $\alpha$ 1-coupled kinase, MAP kinases and cAMP response element binding protein (CREB) in *Ret*<sup>-/-</sup> DRG neurons***

Since the effect of GDNF on  $[Ca^{2+}]_i$  in *Ret*-deficient DRG neurons is specifically dependent on the presence of a GPI-anchored receptor, we focused on a signaling pathway linked to *Ret*-independent GDNF-evoked activation of GFR $\alpha$ 1. GPI-anchored proteins have been shown to be localized to lipid rafts and directly associated with Src family kinases (Berridge, 1998; Harder et al., 1998). There is much evidence suggesting that Src family tyrosine kinases can use PLC $\gamma$  as a substrate (Khare et al., 1997; Berridge, 1998; Harder et al., 1998). We found that GDNF-evoked long-lasting  $Ca^{2+}$  elevation in *Ret*<sup>-/-</sup> neurons was significantly and reversibly inhibited by low doses of selective Src kinase inhibitor PP1 (Fig. 4A, 9 recorded neurons). *In vitro* kinase assay on anti-GFR $\alpha$ 1-precipitated Triton X-100 insoluble lysate from GDNF-stimulated *Ret*<sup>-/-</sup> DRG neurons

revealed a major phosphoprotein of approximately 60 kD (Fig. 4B), that corresponds to the Mr of several Src-type tyrosine kinases (p59 Fyn, pp60 Src and p62 Yes). These ~60 kD bands were absent in the control immunoprecipitation experiments (Fig. 4B).

We further checked the possibility of whether the activation of a GFR $\alpha$ 1-coupled  
5 kinase would lead to phosphorylation of the serine/threonine kinases ERK1 and ERK2 (MAPK) in the absence of Ret, bearing in mind that Src kinases might activate MAPK (Dikic et al., 1996). Using antibodies which recognize phosphorylated MAPK we found that in DRG neurons isolated from E18 Ret<sup>-/-</sup> mice application of GDNF (100 ng/ml) evokes several-fold rapid activation of MAPK (n=3 experiments, Fig. 4C). Exposure of  
10 neurons to neurotrophins, such as BDNF, activates the Ras/ERK/pp90 ribosomal S6 kinase pathway that culminates in CREB phosphorylation (Finkbeiner et al., 1997). We found that CREB phosphorylation in Ser-133 increased substantially already at 5 min after GDNF treatment in Ret<sup>-/-</sup> DRG neurons (Fig. 4D).

## 15 Example 6

### *GDNF stimulates GFR $\alpha$ 1-coupled p62Yes type kinase in SHEP neuroblastoma cells*

We used SHEP human neuroblastoma cells line for further exploration of GDNF-dependent non-Ret signaling. SHEP cells lack Ret mRNA but express GFR $\alpha$ 1 mRNA (Fig.5A) and an ample amount of GFR $\alpha$ 1 protein (data not shown). On average in these  
20 cells, GDNF (100 ng/ml) evoked about a twelve-fold time-dependent increase of a Src-type kinase activity (Fig. 5B) as detected in co-precipitates with anti-GFR $\alpha$ 1 antibodies from Triton X-100 insoluble fractions using a phosphoimager and a TINA program. To identify the particular Src-type kinase, activated by GDNF, we precipitated cell lysate with anti-Yes antibodies and performed the kinase assay thereafter. A major GDNF-stimulated  
25 kinase co-precipitated with GFR $\alpha$ 1 co-migrate with p62Yes kinase (Fig. 5C). Another protein with Mr of about 72-75 kD was also co-precipitated with GFR $\alpha$ 1 antibodies. The nature of this protein is unknown.

**Example 7**

***GDNF activates ERK1/ERK2, CREB and CREB-related protein ATF-1 in SHEP cells.***

In SHEP cells application of GDNF (100 ng/ml) evoked a similar rapid MAPK  
 5 activation as in Ret<sup>-/-</sup> DRG neurons (n=3 experiments, Fig. 6A, compare with Fig. 4C).  
 This fast pattern of MAPK activation in Ret<sup>-/-</sup> E18 DRG neurons and in SHEP  
 neuroblastoma cells is different from the long-lasting elevation of MAPK phosphorylation  
 observed in Ret/GFR $\alpha$ 1 expressing cells (Trupp et al., 1999). The GDNF-dependent  
 phosphorylation of MAPK was almost completely blocked by Src-type kinase inhibitor,  
 10 PP2, already at a low concentration (1  $\mu$ M) (Fig. 6B, n=2 separate experiments). To  
 exclude the possibility that PP2 affected other than Src type kinases, we checked the effect  
 of PP2 on NGF-mediated MAPK activation in PC12 cells. PP2 (0.1 – 10  $\mu$ M) did not  
 affect NGF-dependent activation of MAPK in PC12 (data not shown).

We further investigated whether GDNF affects phosphorylation of CREB and we  
 15 found that GDNF potently induced phosphorylation of CREB in Ser-133 site (Fig. 6C, n=2  
 experiments). Interestingly, GDNF also induced phosphorylation of the CREB-related  
 protein ATF-1 (Fig. 6C). The dynamics of GDNF-induced Ret-independent CREB and  
 ATF-1 phosphorylation was similar to that of GDNF-induced MAPK phosphorylation.

**20 Example 8**

***GDNF induces activation of ERK1/ERK2 in NIH 3T3 fibroblasts via Src type kinases.***

NIH 3T3 fibroblasts stably transfected with GFR $\alpha$ 1 (NIH3T3/pBpGFR $\alpha$ 1) do not  
 express Ret and were therefore used as a non-neuronal cell line for investigating the  
 GDNF-evoked Ret-independent signaling. Src-type kinases can be also co-precipitated  
 25 with GFR $\alpha$ 1 antibodies in these cells (n=2 experiments; Fig. 7A). GDNF (100 ng/ml)  
 again reproducibly increased phosphorylation of MAPK in 3T3NIH/pBpGFR $\alpha$ 1  
 fibroblasts (n=3 experiments, Fig. 7B), although the MAPK activation was delayed in  
 comparison with a rapid activation in Ret<sup>-/-</sup> DRG neurons and in SHEP neuroblastoma  
 cells. Application of PP2 (5  $\mu$ M) again eliminated GDNF-evoked MAPK phosphorylation  
 30 in 3T3NIH/pBpGFR $\alpha$ 1 cells (Fig. 7B, right panel, n=2 experiments). In parental NIH3T3



fibroblasts lacking both Ret and GFR $\alpha$ 1 the application of GDNF (100 ng/ml) either alone or together with a soluble GFR $\alpha$ 1 (GFR $\alpha$ 1/Fc lacking GPI anchor; 1  $\mu$ g/ml) did not evoke any MAPK phosphorylation (data not shown). In the presence of GDNF the soluble GFR $\alpha$ 1 was able to phosphorylate MAPK in Neuro2A neuroblastoma cells lacking  
5 endogenous GFR $\alpha$ 1 but expressing Ret (Fig. 7C).

#### Example 9

##### *GDNF transiently activates PLC $\gamma$ but not JNK in SHEP cells.*

We investigated further whether GDNF can trigger PLC $\gamma$  activation in SHEP cells.  
10 Treatment of SHEP neuroblastoma cells with GDNF (1-100 ng/ml) significantly increased PLC $\gamma$  tyrosine phosphorylation (n=3 experiments, Fig. 8A). The effect was fast, with a significant increase at 1 min, and a return to basal tyrosine phosphorylation levels within 5 min (Fig. 8B). It has been shown that activation of endogenous Ret in PC12 cells as well as activation of transiently or stably expressed Ret in COS-1 cells or NIH3T3 fibroblasts  
15 leads to the phosphorylation of c-Jun NH2-terminal protein kinase (JNK) (Chiariello et al., 1998). In our experiments GDNF (100 ng/ml) did not evoke JNK activation in SHEP cells lacking Ret (n=2 experiments; Fig. 8C).

The foregoing examples are meant to illustrate the invention and are not to be  
20 construed to limit the invention in any way. Those skilled in the art will recognize modifications that are within the spirit and scope of the invention.

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**WHAT IS CLAIMED IS:**

1. A method for identifying a compound that is an agonist of intracellular signaling effected by GPI-anchored receptors in nervous system cells comprising (i) incubating said nervous system cells having GPI-anchored  
5 receptors with a test compound and (ii) determining whether intracellular signaling has been effected in said cells.
2. The method of Claim 1, wherein said nervous system cells express GFR $\alpha$  receptors, but not Ret receptors.
3. The method of Claim 2, wherein said GFR $\alpha$  receptors are GFR $\alpha$ 1  
10 receptors.
4. The method of Claim 1, wherein said nervous system cells are DRG neurons.
5. The method of Claim 4, wherein said DRG neurons are Ret (-/-).
6. The method of Claim 1, wherein said nervous system cells are  
15 neuroblastoma cells.
7. The method of Claim 1, wherein said intracellular signaling is measured as an increase in intracellular Ca<sup>2+</sup> concentration as compared to controls not incubated with said compound.
8. The method of Claim 7 further comprising determining that said test  
20 compound binds to said GPI-anchored receptors.
9. The method of Claim 1, wherein said intracellular signaling is measured as kinase activation.
10. The method of Claim 9, wherein said kinase activation is measured by (i) preparing a cell lysate, (ii) immunoprecipitating the cell lysate with an anti-

GPI-anchored receptor antibody to form an immunoprecipitate, (iii) performing an assay to measure kinase phosphorylation on said immunoprecipitate, and (iv) comparing the results with controls not incubated with said compound.

11. The method of Claim 10, wherein said antibody is anti-GFR $\alpha$ 1.
- 5 12. The method of Claim 9, wherein said kinase is a Src-type kinase.
13. The method of Claim 12, wherein activation of Src-type kinase is measured as activation of MAPK.
14. The method of claim 12, wherein activation of Src-type kinase is measured as activation of CREB.
- 10 15. The method of claim 12, wherein activation of Src-type kinase is measured as PLC $\gamma$  activation.
16. A method for identifying a compound that is an antagonist of intracellular signaling effected by GPI-anchored receptors in nervous system cells comprising (i) incubating said nervous system cells having GPI-anchored  
15 receptors with a test compound in the presence of a sufficient amount of an agonist of said intracellular signaling to effect intracellular signaling, and (iii) comparing the results to controls not incubated with said compound.
17. The method of Claim 16, wherein said nervous system cells express GFR $\alpha$  receptors.
- 20 18. The method of Claim 17, wherein said GFR $\alpha$  receptors are GFR $\alpha$ 1 receptors.
19. The method of Claim 16, wherein said nervous system cells are DRG neurons
20. The method of Claim 19, wherein said DRG neurons are Ret (-/-).

21. The method of Claim 16, wherein said nervous system cells are neuroblastoma cells.
22. The method of Claim 16, wherein the intracellular signaling being measured is an increase intracellular  $\text{Ca}^{2+}$  concentration.
- 5 23. The method of Claim 16, wherein the intracellular signaling being measured is kinase activation.
24. The method of Claim 23, wherein said kinase activation is measured by (i) preparing a cell lysate, (ii) immunoprecipitating the cell lysate with an anti-GPI-anchored receptor antibody to form an immunoprecipitate, (iii) performing  
10 an assay to measure kinase phosphorylation on said immunoprecipitate, and (iv) comparing the results with controls not incubated with said compound.
25. The method of Claim 24, wherein said antibody is anti-GFR $\alpha$ 1.
26. The method of Claim 23, wherein said kinase is a Src-type kinase.
27. The method of Claim 26, wherein said Src-type kinase activation is  
15 measured as activation of MAPK.
28. The method of claim 26, wherein said Src-type kinase activation is measured as CREB activation.
29. The method of claim 26, wherein said Src-type kinase activation is measured as PLC $\gamma$  activation.
- 20 30. A method for identifying a compound that is an agonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling comprising (i) incubating cells that express GFR $\alpha$ 1 receptor, but not Ret receptor, with a test compound and (ii) determining whether intracellular signaling has been effected in said cells.

31. The method of Claim 30, wherein said intracellular signaling is measured as an increase in intracellular  $\text{Ca}^{2+}$  concentration as compared to controls not incubated with said compound.
- 5 32. The method of Claim 31 further comprising determining that said test compound binds to said GPI-anchored receptors.
33. The method of Claim 30, wherein said intracellular signaling is measured as kinase activation.
- 10 34. The method of Claim 33, wherein said kinase activation is measured by (i) preparing a cell lysate, (ii) immunoprecipitating the detergent insoluble fraction of the cell lysate with an anti-GFR $\alpha$ 1 receptor antibody to form an immunoprecipitate, (iii) performing an assay to measure kinase phosphorylation on said immunoprecipitate, and (iv) comparing the results with controls not incubated with said compound.
- 15 35. The method of Claim 33, wherein said kinase is a Src-type kinase.
36. The method of Claim 33, wherein activation of Src-type kinase is measured as activation of MAPK.
37. The method of claim 33, wherein activation of Src-type kinase is measured as activation of CREB.
- 20 38. The method of claim 33, wherein activation of Src-type kinase is measured as PLC $\gamma$  activation.
39. A method for identifying a compound that is an antagonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling comprising (i) incubating cells that express GFR $\alpha$ 1 receptor, but not Ret receptor, with a test compound in the presence of a sufficient amount of an agonist of said intracellular signaling

to effect intracellular signaling, and (iii) comparing the results to controls not incubated with said compound.

40. The method of Claim 39, wherein said agonist is GDNF.

5 41. The method of Claim 39, wherein said intracellular signaling is measured as an increase in intracellular  $\text{Ca}^{2+}$  concentration as compared to controls not incubated with said compound.

42. The method of Claim 39 further comprising determining that said test compound binds to said GPI-anchored receptors.

10 43. The method of Claim 39, wherein said intracellular signaling is measured as kinase activation.

44. The method of Claim 43, wherein said kinase activation is measured by (i) preparing a cell lysate, (ii) immunoprecipitating the detergent insoluble fraction of the cell lysate with an anti-GFR $\alpha$ 1 receptor antibody to form an immunoprecipitate, (iii) performing an assay to measure kinase phosphorylation on said immunoprecipitate, and (iv) comparing the results with controls not incubated with said compound.

15

45. The method of Claim 43, wherein said kinase is a Src-type kinase.

46. The method of Claim 43, wherein activation of Src-type kinase is measured as activation of MAPK.

20 47. The method of claim 43, wherein activation of Src-type kinase is measured as activation of CREB.

48. The method of claim 43, wherein activation of Src-type kinase is measured as PLC $\gamma$  activation.



49. A method for identifying a compound which is an agonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling comprising (i) incubating cells which express GFR $\alpha$ 1 receptor, but not Ret receptor, with a test compound (ii) determining whether an increase in intracellular Ca<sup>2+</sup> concentration is effected in said cells as compared to controls not incubated with said compound.
50. The method of Claim 49 further comprising determining that said test compound binds to GFR $\alpha$ 1 receptors.
51. The method of Claim 49, wherein said cells are DRG Ret (-/-) neurons.
52. The method of Claim 49, wherein said cells are transformed cells.
53. The method of Claim 49, wherein said cells are neuroblastoma cells.
54. A method for identifying a compound which is an antagonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling comprising (i) incubating cells which express GFR $\alpha$ 1 receptors, but not Ret receptors, with a compound to be tested in the presence of a sufficient amount of an agonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling to cause an increase in intracellular Ca<sup>2+</sup> concentration, and (ii) determining whether a decrease in intracellular Ca<sup>2+</sup> concentration is effected, as compared with controls performed without said compound to be tested.
55. The method of Claim 54, wherein said agonist is GDNF.
56. The method of Claim 54, wherein said cells are DRG Ret (-/-) neurons.
57. The method of Claim 54, wherein said cells are transformed cells.
58. The method of Claim 54, wherein said cells are neuroblastoma cells.
59. A method for identifying a compound which is an agonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling comprising (i) incubating

5 cells which express GFR $\alpha$ 1, but not Ret, with the compound to be tested, (ii) preparing a cell lysate, (iii) immunoprecipitating the detergent insoluble fraction of the cell lysate with anti-GFR $\alpha$ 1 antibodies to form an immunoprecipitate, and (iv) performing an assay for measuring kinase phosphorylation on said immunoprecipitate.

60. The method of Claim 59, wherein said cells are DRG Ret (-/-) neurons.

61. The method of Claim 59, wherein said cells are transformed cells.

62. The method of Claim 59 wherein said cells are neuroblastoma cells.

10 63. A method for identifying a compound which is an antagonist of the GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling comprising (i) incubating cells which express GFR $\alpha$ 1, but not Ret, with the compound to be tested in the presence of a sufficient amount of an agonist of said intracellular signaling to effect kinase phosphorylation ii) preparing a cell lysate, (iii) immunoprecipitating the detergent insoluble fraction of the cell lysate with anti-  
15 GFR $\alpha$ 1 antibodies to form an immunoprecipitate, (iv) performing an assay for measuring kinase phosphorylation on said immunoprecipitate, and (v) comparing the results of said assay to those achieved in control experiments performed in the absence of said compound to be tested.

64. The method of Claim 63 wherein said agonist is GDNF.

20 65. The method of Claim 63, wherein said cells are DRG Ret (-/-) neurons.

66. The method of Claim 63, wherein said cells are transformed cells.

67. The method of Claim 63, wherein said cells are neuroblastoma cells.

25 68. A method for identifying a compound which is an agonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling comprising (i) incubating cells which express GFR $\alpha$ 1, but not Ret, with a compound to be tested, and (ii)

determining whether activation of Src-type kinase is effected, as compared with controls not incubated with said compound.

69. The method of Claim 68, wherein the Src-type kinase is selected from the group consisting of Fyn, c-Src, and Yes.
- 5 70. The method of Claim 68, wherein Src-type kinase activation is determined by measuring phosphorylation of MAPK.
71. The method of Claim 68, wherein Src-type kinase activation is determined by measuring phosphorylation of CREB.
72. The method of Claim 68, wherein said cells are DRG Ret (-/-) neurons.
- 10 73. The method of Claim 68, wherein said cells are transformed cells.
74. The method of Claims 68, wherein said cells are neuroblastoma cells.
75. A method for identifying a compound which is an antagonist of the GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling pathway comprising (i) incubating cells which express GFR $\alpha$ 1, but not Ret, with a compound to be tested in the presence of a sufficient amount of an agonist of said pathway to cause activation of Src-type kinase and (ii) determining whether said compound effects a decrease in Src-type kinase activation, as compared with controls not incubated with said compound.
- 15
76. The method of Claim 75 wherein the Src-type kinase is selected from the group consisting of Fyn, c-Src and Yes.
- 20
77. The method of Claim 75, wherein Src-type kinase activation is determined by measuring phosphorylation of MAPK.
78. The method of Claim 75, wherein Src-type kinase activation is determined by measuring phosphorylation of CREB.

79. The method of Claim 75, wherein said agonist is GDNF.
80. The method of Claim 75, wherein said cells are DRG Ret (-/-) neurons.
81. The method of Claim 75, wherein said cells are transformed cells.
82. The method of Claim 75, wherein said cells are neuroblastoma cells.
- 5 83. A method for identifying a compound which is an agonist of intracellular signaling effected by GFR $\alpha$  receptors comprising (i) incubating lipid rafts prepared from cells having GFR $\alpha$  receptors with said compound and (ii) determining whether Src-type kinase is activated as compared to controls not incubated with said compound.
- 10 84. The method of Claim 83 wherein the Src-type kinase is selected from the group consisting of Fyn, c-Src and Yes.
85. The method of Claim 83, wherein Src-type kinase activation is determined by measuring phosphorylation of MAPK.
86. The method of Claim 83, wherein Src-type kinase activation is  
15 determined by measuring phosphorylation of CREB.
87. A method for identifying a compound which is an antagonist of intracellular signaling effected by GFR $\alpha$  receptors comprising (i) incubating lipid rafts prepared from cells having GFR $\alpha$  receptors with said compound in the presence of a sufficient amount of an agonist of the GFR $\alpha$ -dependent, Ret-  
20 independent intracellular signaling pathway to activate Src-type kinases and (ii) comparing the results to control experiments performed in the absence of said compound.
88. The method of Claim 87 wherein the Src-type kinase is selected from the group consisting of Fyn, c-Src and Yes.

89. The method of Claim 87, wherein Src-type kinase activation is determined by measuring phosphorylation of MAPK.
90. The method of Claim 87, wherein Src-type kinase activation is determined by measuring phosphorylation of CREB.
- 5 91. The method of Claim 87, wherein said agonist is GDNF.
92. A method for effecting a cellular response in nervous system cells comprising administering an effective amount of an agonist or antagonist of GPI-anchored intracellular signaling.
- 10 93. The method of Claim 92, wherein said nervous system cells are motor neurons.
94. The method of Claim 92, wherein said nervous system cells are sensory neurons.
95. The method of Claim 92, wherein said sensory neurons are auditory neurons.
- 15 96. The method of Claim 92, wherein said nervous system cells are glial cells.
97. The method of claims 92-97 wherein said agonist or antagonist of GPI-anchored, intracellular signaling is an agonist or antagonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling.
- 20 98. A method for treating neuronal disease comprising administering an effective amount of an agent which is an agonist of GPI-anchored intracellular signaling.

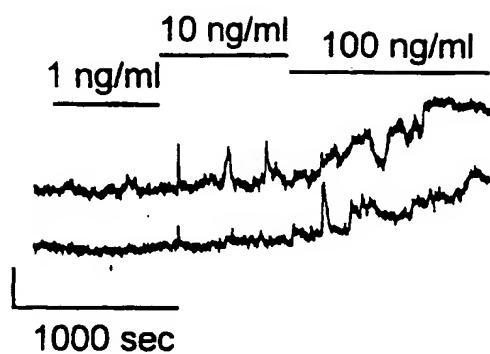
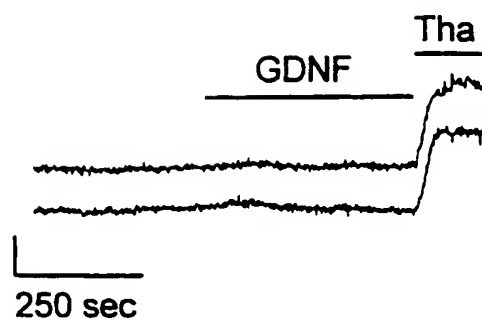
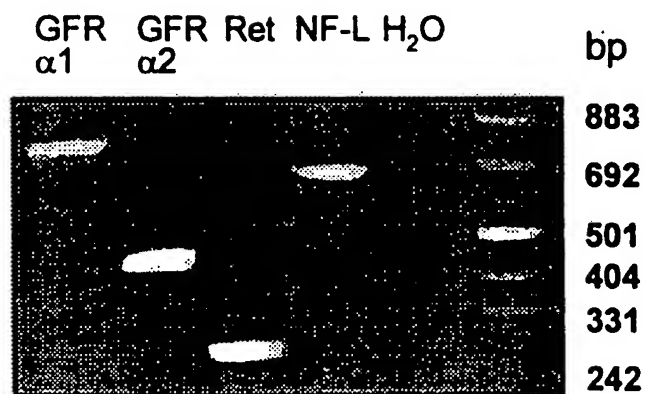
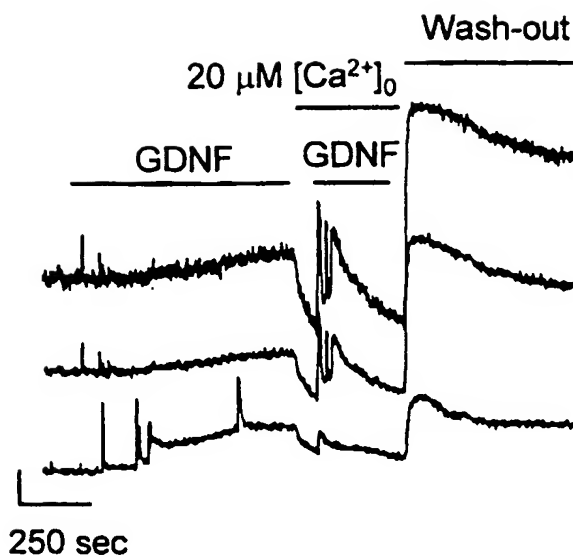
99. The method of Claim 98, wherein said agonist of GPI-anchored intracellular signaling is an agonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling.
100. A method for treating neuronal disease comprising administering an effective amount of an agent which is an antagonist of GPI-anchored intracellular signaling.
101. The method of Claim 100, wherein said antagonist of GPI-anchored, intracellular signaling is an antagonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling.
102. A method for treating neuronal disease comprising administering an effective amount of an agent that is an agonist of both Ret-dependent and GPI-anchored, Ret-independent intracellular signaling.
103. The method of Claim 102, wherein said agonist of GPI-anchored, Ret-independent intracellular signaling is an agonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling.
104. A method for treating neuronal disease comprising administering an effective amount of an agent that is an antagonist of both Ret-dependent and GPI-anchored, Ret-independent intracellular signaling.
105. The method of Claim 104, wherein said antagonist of GPI-anchored, Ret-independent intracellular signaling is an antagonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling.
106. A method for treating neuronal disease comprising administering an effective amount of an agent or agents that are agonists of Ret-dependent intracellular signaling and antagonists of GPI-anchored, Ret-independent intracellular signaling.

107. The method of Claim 106, wherein said antagonists of GPI-anchored, Ret independent intracellular signaling are antagonists of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling.
- 5 108. A method for treating neuronal disease comprising administering an effective amount of an agent or agents that are antagonists of Ret-dependent intracellular signaling and agonists of GPI-anchored, Ret-independent intracellular signaling.
- 10 109. The method of Claim 108, wherein said agonists of GPI-anchored, Ret independent intracellular signaling are agonists of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling.
110. A method for protecting auditory neurons comprising administering an effective amount of an agent which is an agonist of GPI-anchored intracellular signaling.
- 15 111. A method for preventing memory loss comprising administering an effective amount of an agent which is an agonist of GPI-anchored intracellular signaling.
112. A method of improving learning ability comprising administering an effective amount of an agent which is an agonist of GPI-anchored intracellular signaling.
- 20 113. A method for treating epilepsy comprising administering an effective amount of an agent which is an agonist of GPI-anchored intracellular signaling.
114. A method for treating Parkinson's Disease comprising administering an effective amount of an agent which is an agonist of GPI-anchored, intracellular signaling.

115. The method of Claims 110-114 wherein said agonist of GPI-anchored, intracellular signaling is an agonist of GFR $\alpha$ 1-dependent, Ret-independent intracellular signaling.



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*FIG. 1A**FIG. 1B**FIG. 1C**FIG. 1G*

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FIG. 1D

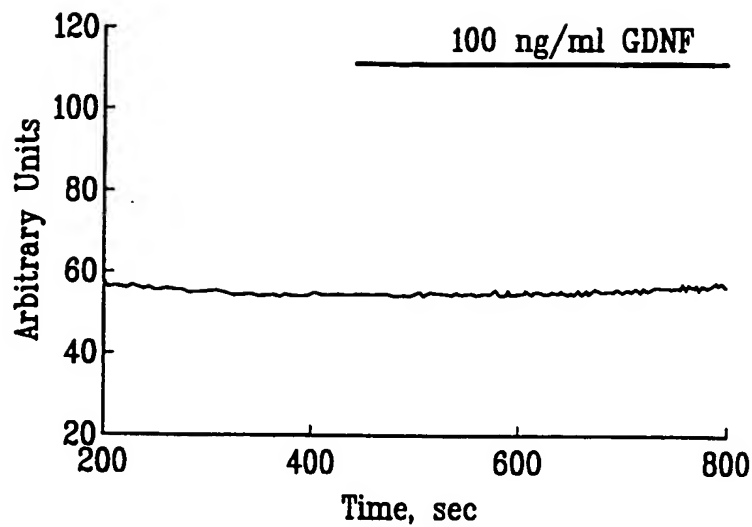


FIG. 1F

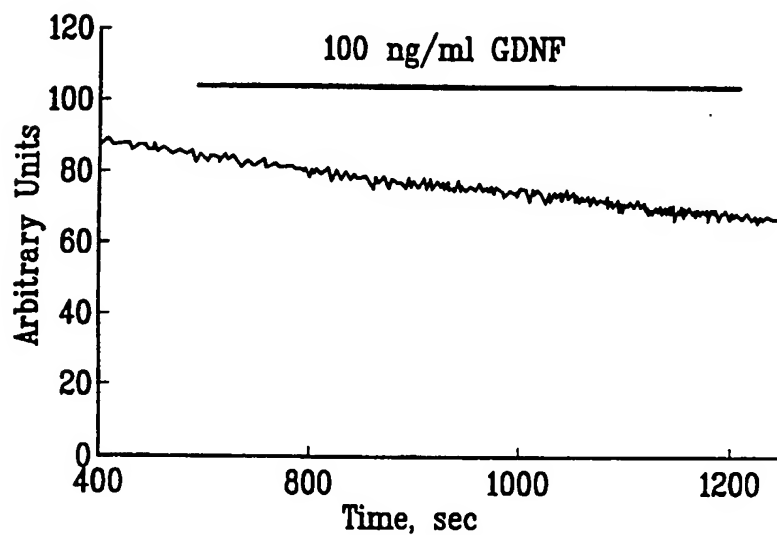
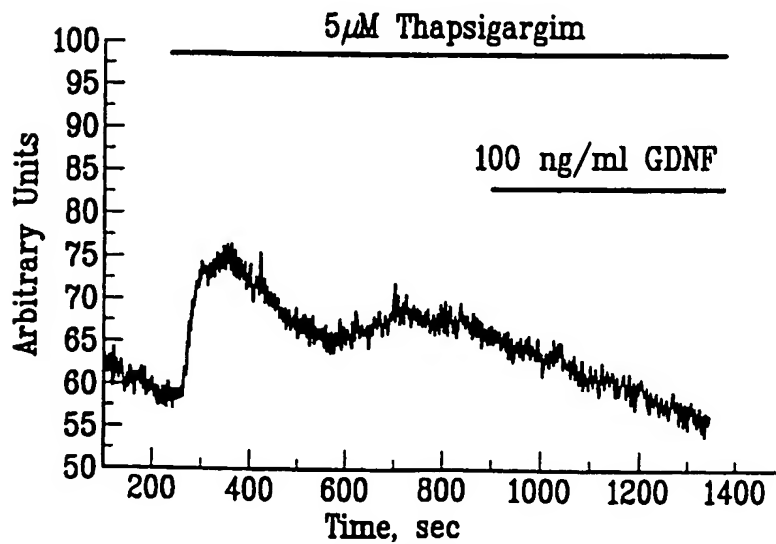


FIG. 1E



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FIG. 2A

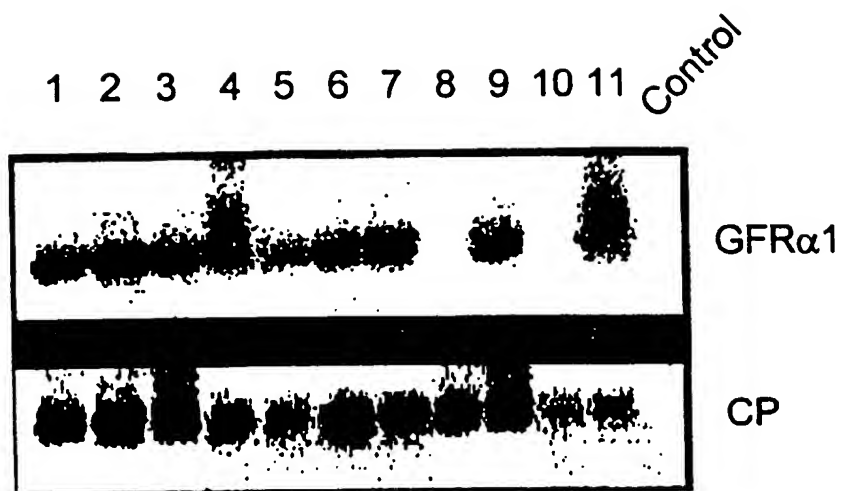


FIG. 2B

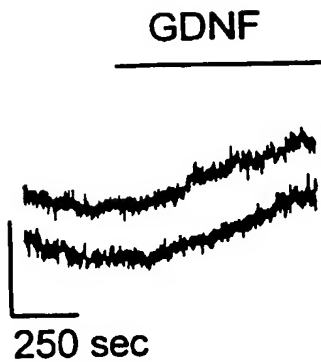


FIG. 2C

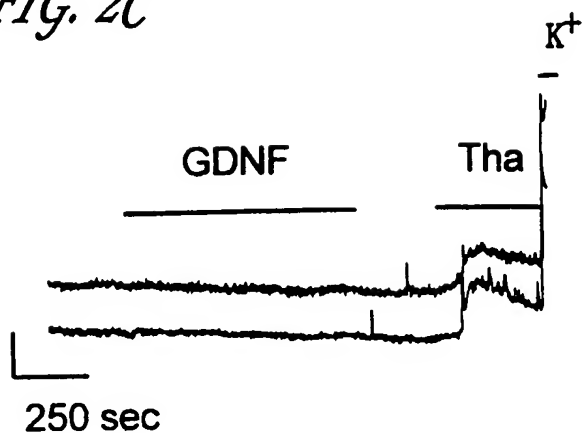
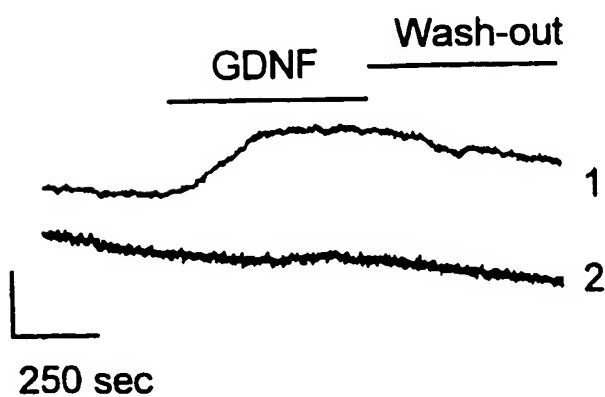
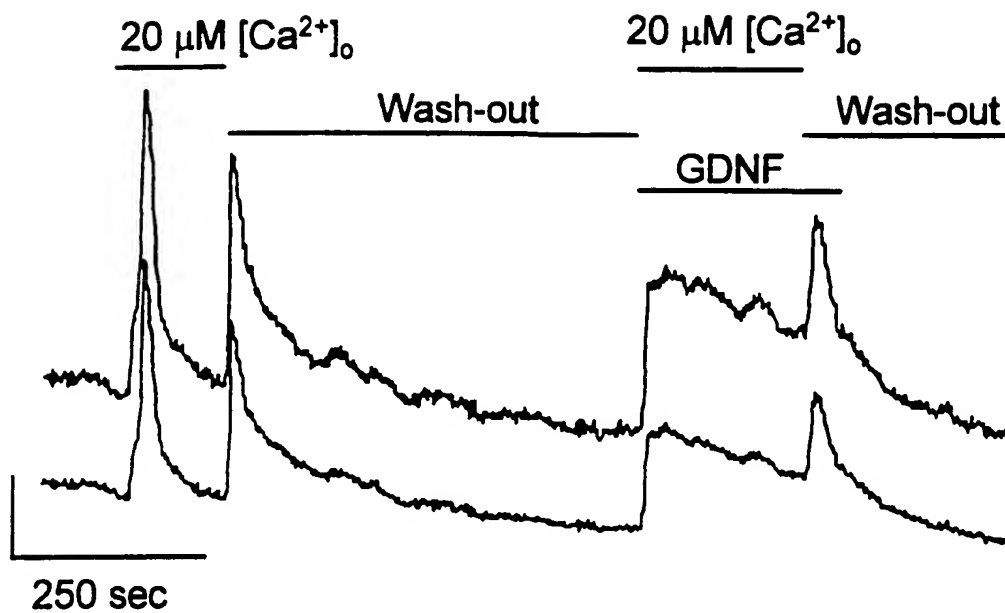
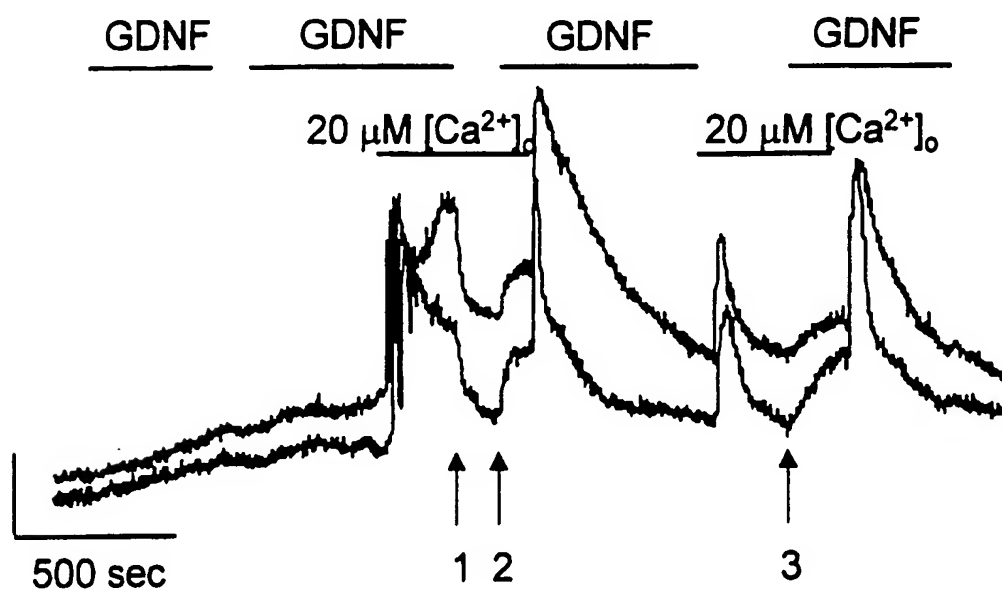


FIG. 2D



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*FIG. 3A**FIG. 3B*

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FIG. 3C

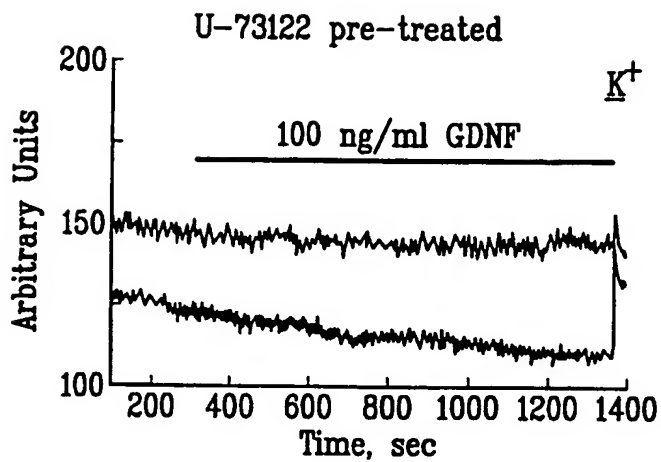


FIG. 3D

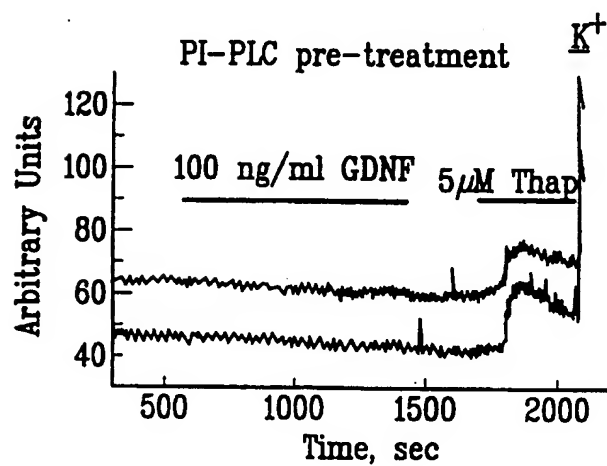
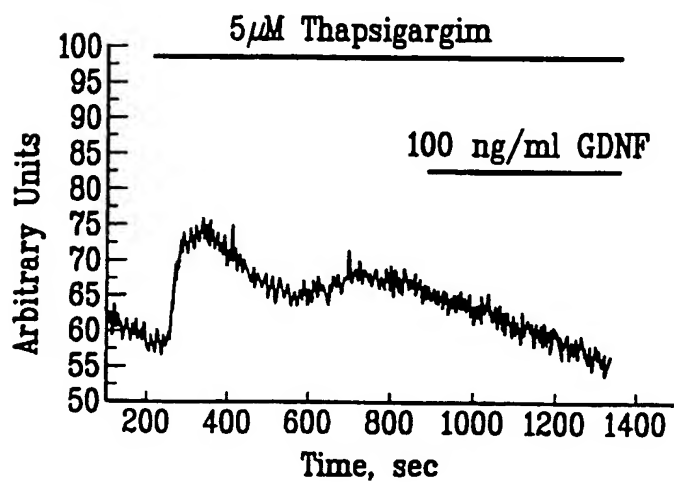
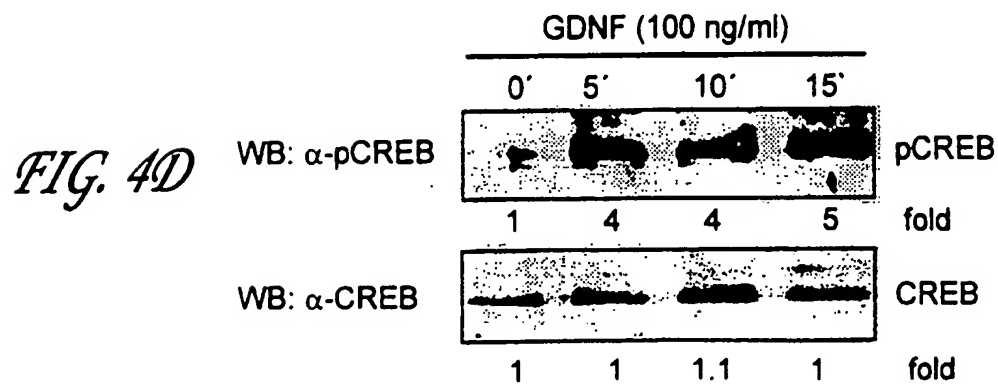
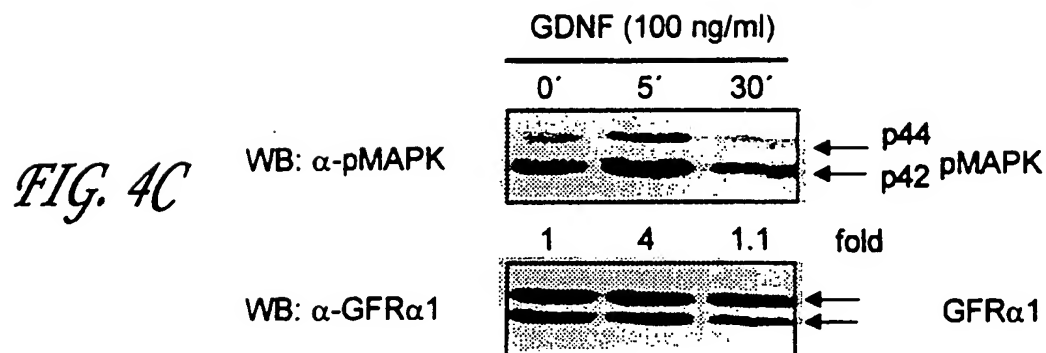
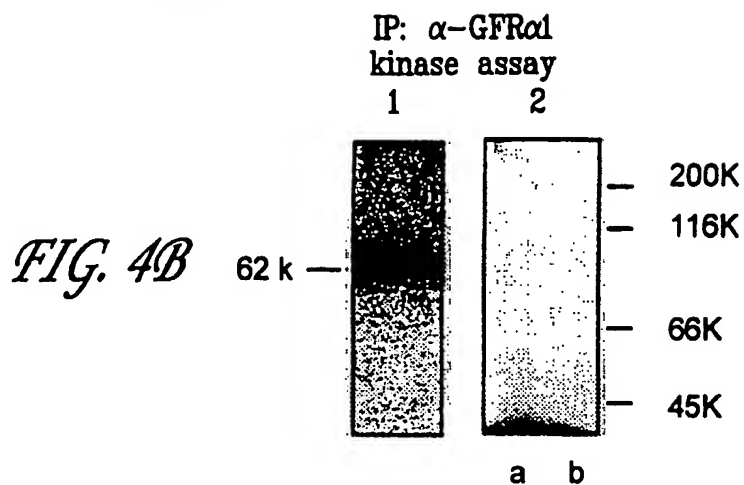
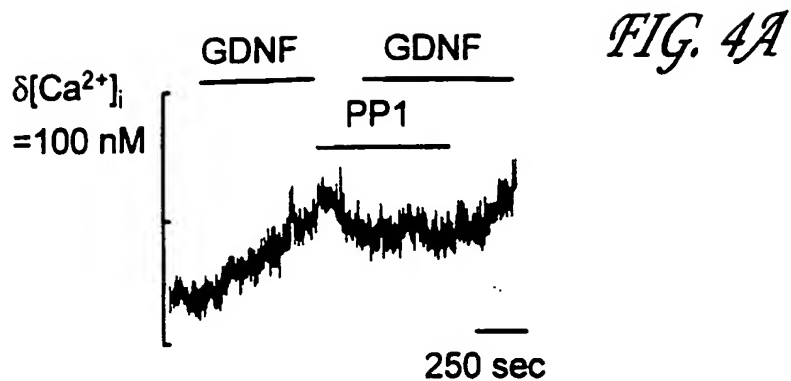


FIG. 3E



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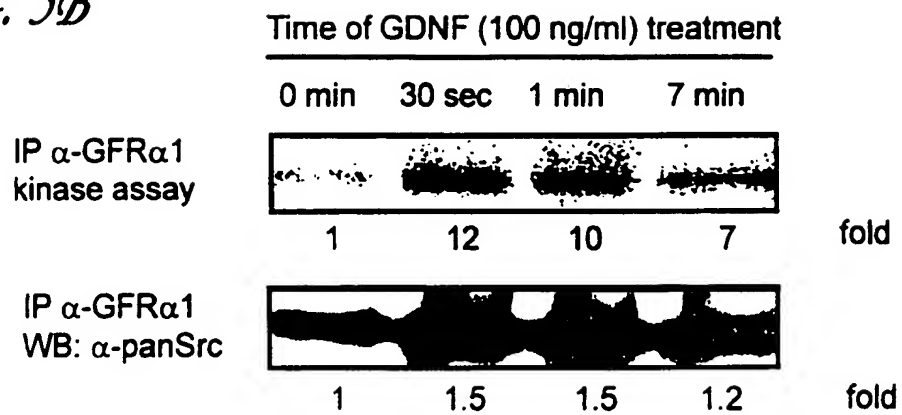


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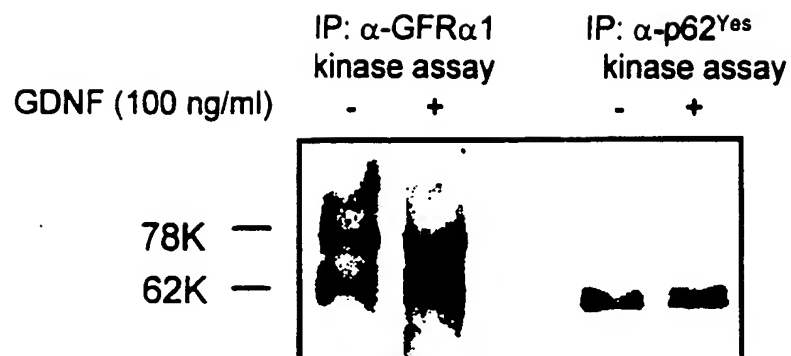
*FIG. 5A*



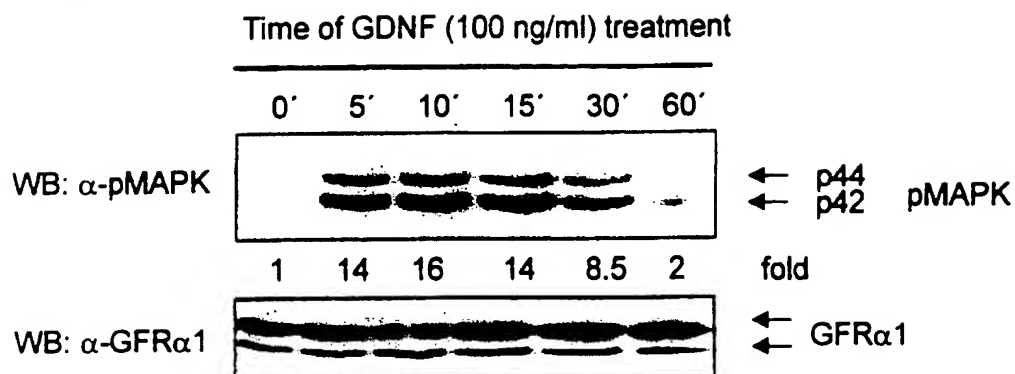
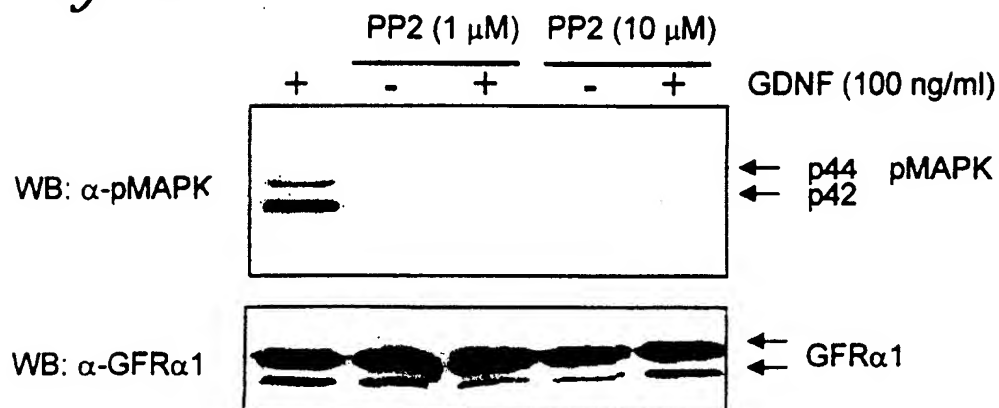
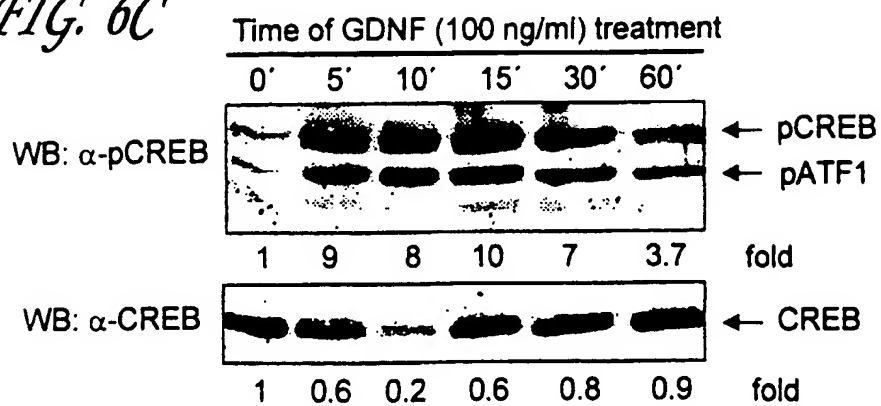
*FIG. 5B*



*FIG. 5C*



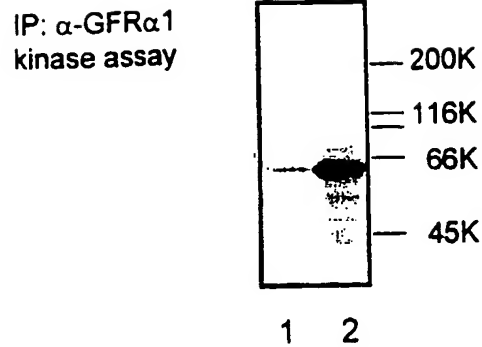
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*FIG. 6A**FIG. 6B**FIG. 6C*



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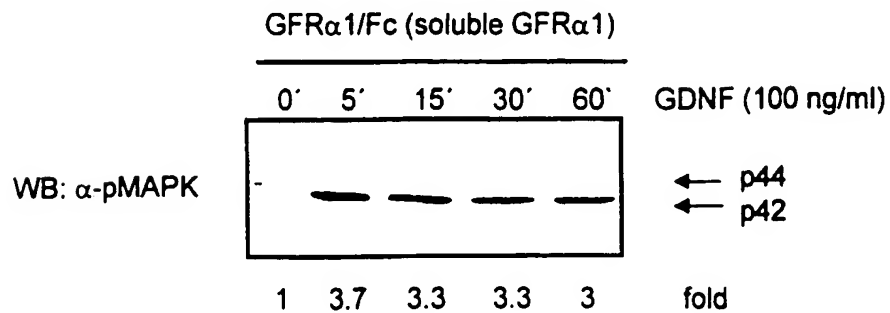
*FIG. 7A*



*FIG. 7B*

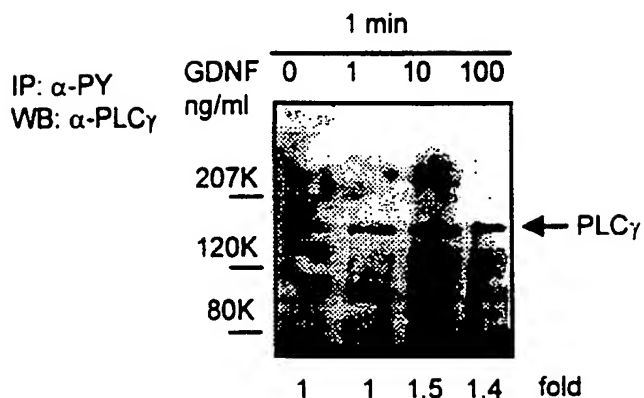


*FIG. 7C*

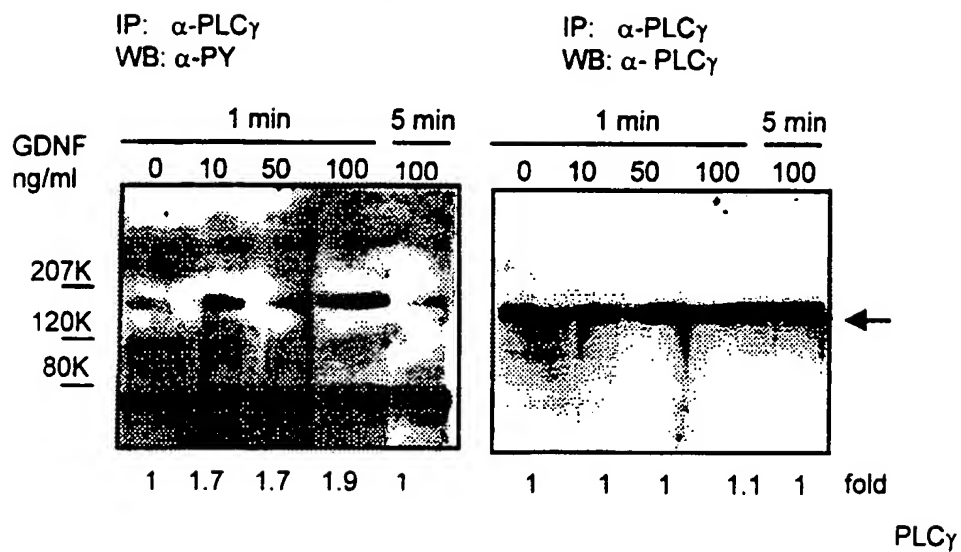


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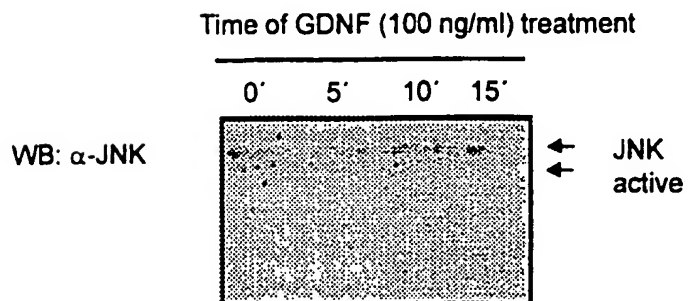
*FIG. 8A*

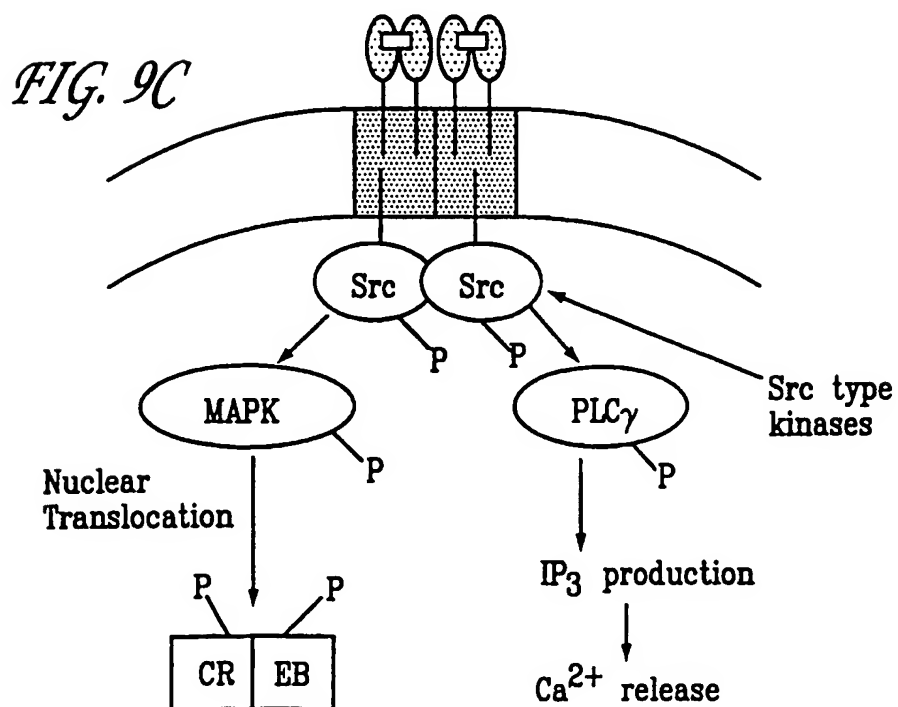
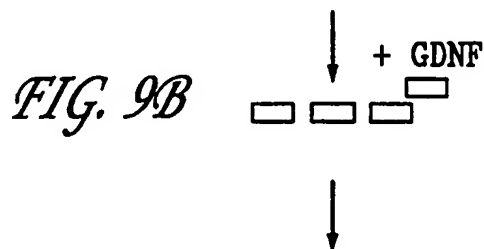
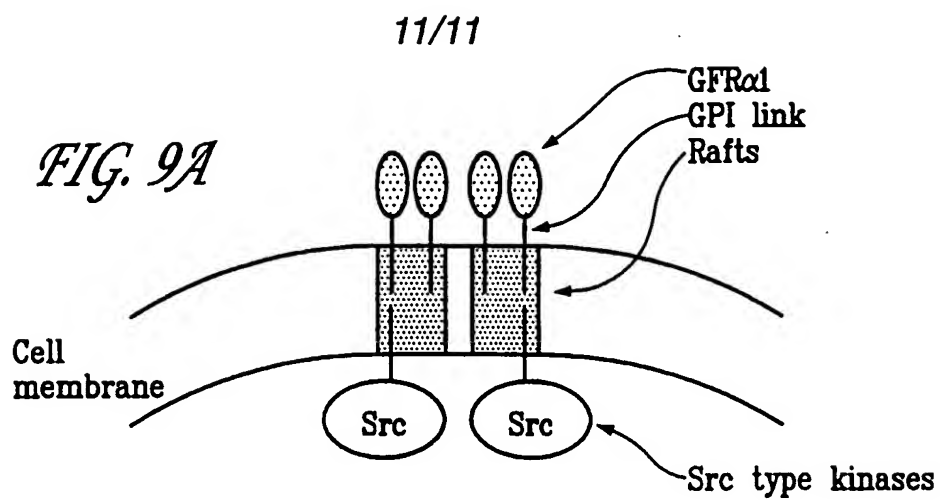


*FIG. 8B*



*FIG. 8C*





## INTERNATIONAL SEARCH REPORT

Inter. Appl. Application No

PCT/IB 99/01681

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 G01N33/566

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	POTERYAEV D. ET AL.: "GDNF triggers a novel Ret-independent SRC kinase family-coupled signaling via a GPI-linked GDNF receptor alpha1" FEBS LETTERS, vol. 463, 1999, pages 63-66, XP002132168 the whole document	1-97
P,X	TRUPP M. ET AL.: "Ret-dependent and -independent mechanisms of glial cell line-derived neurotrophic factor signaling in neuronal cells" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 30, 23 July 1999 (1999-07-23), pages 20885-20894, XP000889729 the whole document	1-97

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

7 March 2000

Date of mailing of the international search report

27/03/2000

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/IB 99/01681

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 18240 A (LINDAHL MARIA ; SAARMA MART (FI); SARIOLA HANNU (FI); SUVANTO PETRO) 22 May 1997 (1997-05-22) claims 2,10	1-97
X	WO 98 36072 A (GENENTECH INC) 20 August 1998 (1998-08-20) abstract page 1, line 1,2,13-17 claim 11	1-97
X	TREANOR J.J.S. ET AL.: "Characterization of a multicomponent receptor for GDNF" NATURE, vol. 382, 4 July 1996 (1996-07-04), pages 80-83, XP002132170 the whole document	1-97
X	JING S ET AL: "GDNF-INDUCED ACTIVATION OF THE RET PROTEIN TYROSINE KINASE IS MEDIATED BY GDNFR-ALPHA, A NOVEL RECEPTOR FOR GDNF" CELL,US,CELL PRESS, CAMBRIDGE, NA, vol. 85, 28 June 1996 (1996-06-28), page 111 XP002065819 ISSN: 0092-8674 the whole document	1-97

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 99/01681

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 98-115  
because they relate to subject matter not required to be searched by this Authority, namely:  
Claims 98-115 are related to methods of treatment of a human body by therapy, therefore they are excluded from patentability.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

**PCT/IB 99/01681**

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
W0 9718240 A	22-05-1997	AU 1158897 A	05-06-1997
		CA 2237641 A	22-05-1997
		EP 0879247 A	25-11-1998
		NO 982141 A	13-07-1998
		NZ 324511 A	29-09-1999
W0 9836072 A	20-08-1998	AU 6175098 A	08-09-1998

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